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An Ancient Role for TRPA1 in Cold Nociception

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An Ancient Role for *TrpA1* in Cold Nociception

by

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Under the Direction of Daniel N. Cox, PhD

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ABSTRACT

Thermosensory nociception alerts organisms to potential environmental dangers, thereby serving as a protective mechanism for driving adaptive behavioral responses to safeguard against incipient damage. Transient receptor potential (TRP) channels play a key role in thermosensation and some can be activated directly or indirectly by changes in temperature. Protostome, and many deuterostome, TRPA1s have been associated with high-temperature sensing. For example, *Drosophila* TRPA1 is known to function in Class IV (CIV) polymodal nociceptor neurons for high heat (as well as mechanical and chemical) nociception. Conversely, some deuterostome TRPA1s (including those of mice and humans) have been identified as noxious cold sensitive. However, little is known regarding how TRPs mechanistically function in regulating noxious cold detection and whether there is molecular conservation across phyla.

Our previous studies identified Class III (CIII) multidendritic (md) sensory neurons as cold nociceptors that are required for noxious cold-evoked contraction (CT) behavior in larvae. We further demonstrated via CIII-specific neurogenomic studies that TRPA1 is enriched in these neurons, suggesting it may contribute to noxious cold sensing. In this work, analyses of multiple *TrpA1* whole-animal mutants revealed severe impairment of cold-evoked CT behavior, which we likewise observed with CIII-specific *TrpA1* knockdown. Behavioral studies of *TrpA1* mutations revealed isoform-specific requirements for noxious cold sensing that are distinct from isoforms linked to noxious heat sensing via CIV neurons. Electrophysiological recordings demonstrate that TRPA1 is required in CIII neurons for cold-evoked neural activity. TrpA1 appears to function in cold-evoked sensory transduction, and CIII-specific expression of *TrpA1* rescued nocifensive behavior in whole animal *TrpA1* mutants. Further, behavioral deficits were also rescued by ectopic expression of human *TrpA1*. Moreover, ectopic expression studies reveal TrpA1 is sufficient to

confer cold sensitivity on otherwise non-cold-sensing neurons. Collectively, these findings shed new light on evolutionarily ancient properties of TRPA1 in thermal sensing and raise interesting questions regarding the molecular and mechanistic underpinnings of TRPA1 polymodality.

INDEX WORDS: TRPA1, thermoTRP, ANKTM1, Cold nociception, Pain, Hyperalgesia; Gr28b

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Conceptualization, Daniel N. Cox and Nathaniel J. Himmel; Methodology, D.N.C., N.J.H., Atit A. Patel, Anna Rader, and Akira Sakurai; Software, A.A.P.; Investigation, N.J.H., Mariana Holguin-Lopez, Inara Jawed, Erin Lottes, Romee Maitra, Dusty Moon, A.R., and A.S.; Resources, Marco Gallio, Y.N. Jan, W. Daniel Tracey, and Yang Xiang; Visualization, N.J.H. and A.S.; Funding Acquisition, D.N.C. Additional funding was provided by Brains and Behavior Fellowships (N.J.H., J.M.L., A.A.P.) and an Initiative for Maximizing Student Development Fellowship (M.H.L). I would like to acknowledge Dr. Jordan Hamm for his invaluable assistance in data analysis.

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
TrpA1	Transient Receptor Potential Channel Ankyrin subtype 1
ANKTM1	ankyrin-like protein with transmembrane domains 1 [=TRPA1]
CIII	class III multidendritic neuron
CIV	class IV multidendritic neuron
CT	contraction
PER	proboscis extension response
$TrpA^{AmHs}$	Hymenoptera-specific TRPA
SEP	standard error of proportion
ROS	reactive oxygen species
Duox	dual oxidase
SOD	superoxide dismutase
Cat	catalase

1 INTRODUCTION

1.1 Nociception across animal taxa

All animals encounter a variety of potentially harmful environmental factors, which include noxious chemical, mechanical, and thermal stimuli. Nociception, observed throughout the metazoans, is the ability to detect and respond to these potentially damaging stimuli (Basso & Altier, 2017; Himmel et al., 2017; Julius, 2013). Nocifensive behaviors typically involve a sensorimotor circuit which encodes and interprets sensory inputs and elicits a behavior that permits the animal to evade or minimize damage (Basbaum et al., 2009; Himmel & Cox, 2020; Tracey, 2017). Noxious stimuli of various modalities are received by high-threshold sensory neurons termed nociceptors (*e.g.*, mammalian C and Aδ fibers) (Ji et al., 2008; Kobayashi et al., 2005), which communicate the signal of threat downstream in the circuit (Chin & Tracey, 2017; Tracey, 2017). Disordered nociception results in hyperalgesia, abnormally hypersensitized perception of noxious stimuli, or in allodynia, the sensation of pain caused by innocuous stimuli (Y. Luo et al., 2021). Collectively, these are termed neuropathic pain, and present an enormous and costly burden on patients and on the whole of society (Calvo et al., 2019; Rice et al., 2016). Therefore, an elucidation of the mechanisms of nociception is essential to understanding the etiology of, and identifying potential therapies for, the conditions associated with it.

1.2 *TrpA1* is a polymodal mediator of cold nociception

Transient receptor potential (TRP) channels are non-specific cation channels expressed in a broad variety of tissue types, including in certain nociceptor neurons (Ji et al., 2008; Kobayashi et al., 2005; Zielińska et al., 2015). Upon gating, they permit the influx of Ca^{2+} and subsequent depolarization of the cell membrane (Gees et al., 2010). The TRP subfamily A member 1 (*TrpA1*)

is essential for proper nociception in both deutersostomes and protostomes (Julius, 2013; Venkatachalam & Montell, 2007). TRPA1 is itself a polymodal channel, responding to noxious mechanical, chemical, and thermal stimuli (Himmel et al., 2019; Moparthi & Zygmunt, 2020). Regarding its role in high heat nociception, *TrpA1* has been extensively studied in both vertebrates and invertebrates (Barbagallo & Garrity, 2015; Basbaum et al., 2009; Cordero-Morales et al., 2011; Islas, 2018; Laursen et al., 2015). However, relatively little is known regarding how *TrpA1* mechanistically functions in regulating noxious cold detection, and whether there is molecular conservation across phyla (Chen et al., 2013; Kwan & Corey, 2009; Moparthi et al., 2014). TRPA1 channels are highly conserved and thought to have functioned in chemical sensing since at least before the protostome-deuterostome split (>550 mya) (Dong et al., 2016; Himmel et al., 2019; Kang et al., 2010; Zhao et al., 2020), yet it remains unknown whether TRPA1 thermal sensing is equally ancient (Peng et al., 2015).

TrpA1 thermal sensitivity was first described in Chinese hamster ovary (CHO) cells expressing the recombinant mouse ortholog, where it was reported to be a noxious cold (≤17°C) sensor (Story et al., 2003). However, the thermal sensitivity of mammalian *TrpA1* has been the subject of controversy, with numerous groups reporting apparently contradictory findings. One investigation using *in vivo* single fiber recording failed to detect noxious-cold-evoked responses in *TrpA1*-expressing primary afferents of rat paws (Dunham et al., 2010), while another group reported that *TrpA1* mutant mice exhibited significant loss of nocifensive response to cold plate paw stimulus (Kwan et al., 2006). Further studies in human embryonic kidney (HEK) 293 cells reported that both rat and mouse *TrpA1*s are cold sensitive, while those of rhesus monkeys and humans are thermally insensitive (Chen et al., 2013). One study of human *TrpA1* in CHO cells found it to be activated by noxious cold stimulus (Bandell et al., 2004), while another investigation

in HEK293 cells found no such link to cold (Zurborg et al., 2007). Recent studies employing single-channel electrophysiological recordings of *TrpA1* reconstituted in lipid bilayers have confirmed that human *TrpA1* is intrinsically cold and high heat sensitive (Moparthi et al., 2014). Further, numerous mutations of human *TrpA1* have been implicated in cold neuropathies (Naert et al., 2020). Whether mammalian (or any other) *TrpA1* is directly gated by cold under physiological conditions remains contentious (Manolache et al., 2021). However, collectively, these reports suggest that it does function in the mammalian cold nociceptive circuit, regardless of gating mechanism.

It has commonly been assumed that ancestral TRPA1 was high heat sensitive and cold insensitive—and that there was an evolutionary divergence between the thermal modality of TRPA1 in certain lineages (Chen, 2015). This interpretation resulted from the fact that all known protostome TRPA1s, as well as those of many vertebrates, including fish, amphibians, reptiles, and birds have been associated with noxious high temperatures (Hoffstaetter et al., 2018; Laursen et al., 2015), while only mammalian and zebrafish TRPA1s have been demonstrated to function in noxious cold detection (Chen et al., 2013; Kwan & Corey, 2009; Moparthi et al., 2014; Oda et al., 2016) (**Fig. 2-1**). Tests of this hypothesis, however, are absent from the literature. It is therefore of importance to test whether or not protostome TRPA1s (*e.g.* that of *Drosophila*) function in cold nociception. Moreover, TRPA1 is required for chemical, mechanical, and thermal nociception (Himmel et al., 2017), yet the underpinnings of this polymodality remain unresolved, and there is disagreement concerning the mechanisms of channel gating (*e.g.*, direct or indirect), suggesting that many different mechanisms may be at play across species (Arenas et al., 2017; Doerner et al., 2007). It remains unresolved to what degree different species-specific TRPA1s depend on these different mechanisms; therefore, despite the possibility that TRPA1 cold

sensitivity is widespread, it remains unknown whether or not it has a common origin (Chen, 2015; Laursen et al., 2015). TRPA1 cold sensitivity may be ancient and highly conserved; however, it might also be an example of evolutionary convergence, as it may function via several different mechanisms, which themselves may have evolved independently. (See **Figure 2-1**.)

1.3 *TrpA1* in nociceptive neuropathies

Mutations in *TrpA1* have been associated with numerous pathological states and include neuropathies encompassing several sensory modalities. Two different gain-of-function mutations to *TrpA1* have been identified in humans which result in heightened responses to environmental irritants such as diesel exhaust and coal fly ash. This hypersensitization is correlated with asthmatic episodes in patients carrying these alleles. Conversely, loss-of-function mutations are associated with reduced adverse responses to coal fly ash (Deering-Rice et al., 2015). Numerous additional *TrpA1* polymorphisms have been associated with childhood asthma and neuropathic pain associated with altered mechanonociception (Gallo et al., 2017).

A particular allele (N855S) resulting from a single nucleotide mutation (SNP) substituting guanine for alanine, results in the autosomal dominant familial episodic pain syndrome (FEPS). With onset in infancy, it is characterized by debilitating upper body pain and difficulty breathing, which is triggered by physical stress or exposure to cold thermal stimuli. These patients also exhibit hypersensitivity to allyl isothiocyanate (AITC), the pungent compound found in mustard and wasabi. They possess normal proprioception and innervation of the skin, yet suffer excruciating hypersensitivity to cold (Kremeyer et al., 2010).

Another SNP (710G>A) results in patients being almost entirely insensitive to noxious cold stimuli. Interestingly, they are also more likely to suffer from paradoxical heat sensation, an

allodynia in which innocuous temperatures feel painfully hot (Binder et al., 2011). *TrpA1* is therefore associated with sensory neuropathies in humans via altered chemical, mechanical, and thermal nociception. Elucidation of *TrpA1*'s function in normal nociception may help to shed light on its role in related neuropathic pain.

1.4 *TrpA1* as a mediator of nociception in *Drosophila*

Interestingly, while *TrpA1* has been studied as a mediator of cold nociception in mammals, all arthropod *TrpA1* orthologs with any known thermal sensitivity gate in response to noxious heat (Fowler & Montell, 2013). *Drosophila melanogaster* has provided a powerful, genetically tractable model for the investigation of nociception and the receptors that mediate it (Al-Anzi et al., 2006; Hwang et al., 2012; Jang et al., 2015; Neely et al., 2011). The well-characterized multidendritic (md) sensory neurons of the larva are responsible for mediating nociception in response to stimuli of various modalities. *TrpA1* is known to be expressed in Class IV (CIV) md neurons and has been shown to mediate chemical, mechanical, and high heat nociception (Himmel et al., 2017; Himmel & Cox, 2020; Zhong et al., 2012). *Drosophila* has one identified gene encoding TRPA1, but expresses five distinct isoforms (Gu et al., 2019). These isoforms have shared sequence throughout their C-terminal, intramembranous, and loop domains, but they differ at a segment flanking the Ankyrin repeat domain (ARD) and at the N-terminal domain. Heterologous expression experiments demonstrate that TRPA1-A and TRPA1-D isoforms gate in response to high heat, while TRPA1-B and TRPA1-C isoforms are high-heat-insensitive, although *Drosophila* TRPA1 channel responses to noxious cold temperatures have not been investigated in heterologous systems (Zhong et al., 2012). *TrpA1* isoforms C, D, and E are all expressed in CIV neurons (Gu et al., 2019), suggesting a possible role for the thermally sensitive isoform D in high

heat nociception. However, TRPA1-D is incapable of rescuing nocifensive behavior in *TrpA1* mutant flies, while TRPA1-C is sufficient to do so (Zhong et al., 2012). This intriguing result suggests that TRPA1-C isoform mediates thermal nociception by conferring heat sensitivity on a neuron, while itself being thermally insensitive. The mechanism by which this occurs has yet to be elucidated. Whether any isoform (or complement of isoforms) contributes to cold nociception has not been investigated, thereby leaving questions of mechanism open.

Drosophila larvae also exhibit a distinct behavioral response upon exposure to noxious cold stimuli, which is mediated by CIII md neurons. When challenged with noxious cold temperatures, larvae contract (CT) along the anterior-posterior axis. Three thermally sensitive TRP (thermoTRP) channels, Pkd2, NompC, and Trpm have been identified as necessary for the proper function of this nociceptive circuit (Turner et al., 2016). Microarray data indicate that CIII neurons also express *TrpA1* mRNA, suggesting the possibility that *TrpA1* may also function in the detection of noxious cold in these cells.

1.5 Mechanistic requirement of reactive oxygen species

Reactive oxygen species (ROS) are a normal result of oxidative respiration, produced in the mitochondrion by the reduction of O_2 via the electron transport chain and by NADPH oxidase (Halliwell, 2006). Due to their association with cellular stress and disease states, ROS have historically been viewed solely as harmful (Lushchak, 2014; Ogawa et al., 2016). However, recent research has revealed that ROS functions in numerous critical cell signaling pathways (Oswald et al., 2018; Finkel, 2011; Oswald et al., 2018). The functional requirement of a thermally insensitive TRPA1 channel for high heat nociception in *Drosophila* suggests that the activation of thermal nociceptors is not simply a function of temperature, but requires additional (or alternative) factors.

Recent experiments have demonstrated that noxious heat causes the production of ROS in *Drosophila* larvae (Arenas et al., 2017). Electrophysiology experiments demonstrated that ROS is capable of activating not only *Drosophila* TRPA1, but also orthologs of distantly related species, including humans. Further, TRPA1 orthologs from distantly related species, such as humans, are capable of rescuing high heat nocifensive behavior in *TrpA1* mutant *Drosophila*, suggesting a mechanism conserved across diverse lineages (Arenas et al., 2017). Therefore, elucidation of the evolutionarily conserved mechanisms underlying *TrpA1*-mediated cold nociception in *Drosophila* will help us to identify and further investigate the structures and functions that have been conserved across evolutionary history, and provide insight into human nociception.

Whether noxious cold generates ROS in *Drosophila* has not been explored, nor has a putative relationship between intracellular ROS and TRPA1 gating. This study will test the hypothesis that ROS facilitate nocifensive CT responses in *Drosophila* larvae. If this hypothesis is supported by our work, it will warrant further investigation into whether noxious cold temperatures elicit ROS production, and whether ROS signaling acts in concert with TRPA1 channel activation in CIII neurons to evoke cold nocifensive behaviors.

2 MOLECULAR GENETIC DISSECTION OF THE ROLE OF TRPA1 IN COLD NOCICEPTION

TrpA1 plays an essential molecular role in sensing noxious environmental stimuli, including temperature. Its function in chemical nociception is conserved across taxa, and predates the protostome-deuterostome split (>500 mya) (Himmel et al., 2019), although whether this holds true for noxious cold detection remains unknown (**Fig. 2-1**).

Drosophila melanogaster has provided invaluable insights into the molecular mechanisms underlying thermal nociception and nociceptive sensitization. In *Drosophila* larvae, multidendritic (md) sensory neurons serve a wide range of functions, including the mediation of responses to noxious heat, noxious cold, and mechanosensation. Class IV (CIV) neurons express *TrpA1* and are responsible for mediating chemical, mechanical, and high heat nociception. Moreover, all studies to date in arthropods implicate TRPA1 channels in thermal gating in response to noxious high heat. Conversely, in mammals, including humans, TRPA1 has been implicated in mediating cold nociception. Intriguingly, neurogenomic data from our lab indicate that *TrpA1* expression is enriched in *Drosophila* Class III (CIII) md neurons, which function as cold, but not high heat, nociceptors. We therefore proposed to study the potential mechanistic role(s) of *TrpA1* in detecting noxious cold stimuli.

To elucidate the cellular mechanisms underlying *TrpA1*-mediated cold nociceptive behavior, we have combined neurogenetic perturbations, morphological analyses, neurophysiological studies, dynamic Ca^{2+} imaging, optogenetics, and behavioral assays to characterize cellular mechanisms of *TrpA1*-mediated cold nociception.

2.1 Scientific Premise

TRPA1 is a highly promiscuous channel protein expressed in nociceptive neurons and mediating responses to sensory inputs of multiple modalities, and in many vertebrate species it has been implicated as gating in response to noxious cold temperatures (Fowler & Montell, 2013; Story et al., 2003). In *Drosophila* larval CIV md neurons, *TrpA1* serves a critical role in mediating high heat nociception, but whether *TrpA1* has a conserved function in mediating cold nociception in invertebrates remains an open question.

The *TrpA1* gene encodes mRNAs for five distinct protein isoforms (TRPA1-A, TRPA1-B, TRPA1-C, TRPA1-D, and TRPA1-E). As discussed above, previous studies (Guntur et al., 2017; Gu et al., 2019; J. Luo et al., 2017; Zhong et al., 2012) have revealed that CIV md neuron-mediated high heat nociception in *Drosophila* is dependent upon a specific TRPA1 isoform (TRPA1-C), suggesting that distinct TRPA1 isoforms may contribute to nociception of other modalities.

Neurogenomic data comparing expression levels across md neuron classes in *Drosophila* indicate that *TrpA1* mRNA expression is enriched in CIII neurons (Turner et al., 2016), suggesting a possible functional role in these neurons. Based on these findings, we hypothesized that *TrpA1* may function in mediating cold-evoked nocifensive behavior. To test this hypothesis, we conducted pilot loss of function (LOF) cold nociception behavioral assays on *TrpA1* whole animal mutant alleles and CIII-specific *TrpA1-IR* larvae. These analyses revealed that larvae with disrupted *TrpA1* function exhibited defects in noxious cold-evoked nocifensive behavior thereby implicating TRPA1 in CIII md neuron mediated cold nociception. While providing evidence that TRPA1 cold sensitivity is present in protostomes is an important contribution in and of itself (and suggests that TRPA1 cold sensitivity pre-dates the protostome-deuterostome split), this work sets

the foundation by which several competing hypotheses can be tested, and may eventually help to elucidate the evolutionary history of *TrpA1* cold sensitivity.

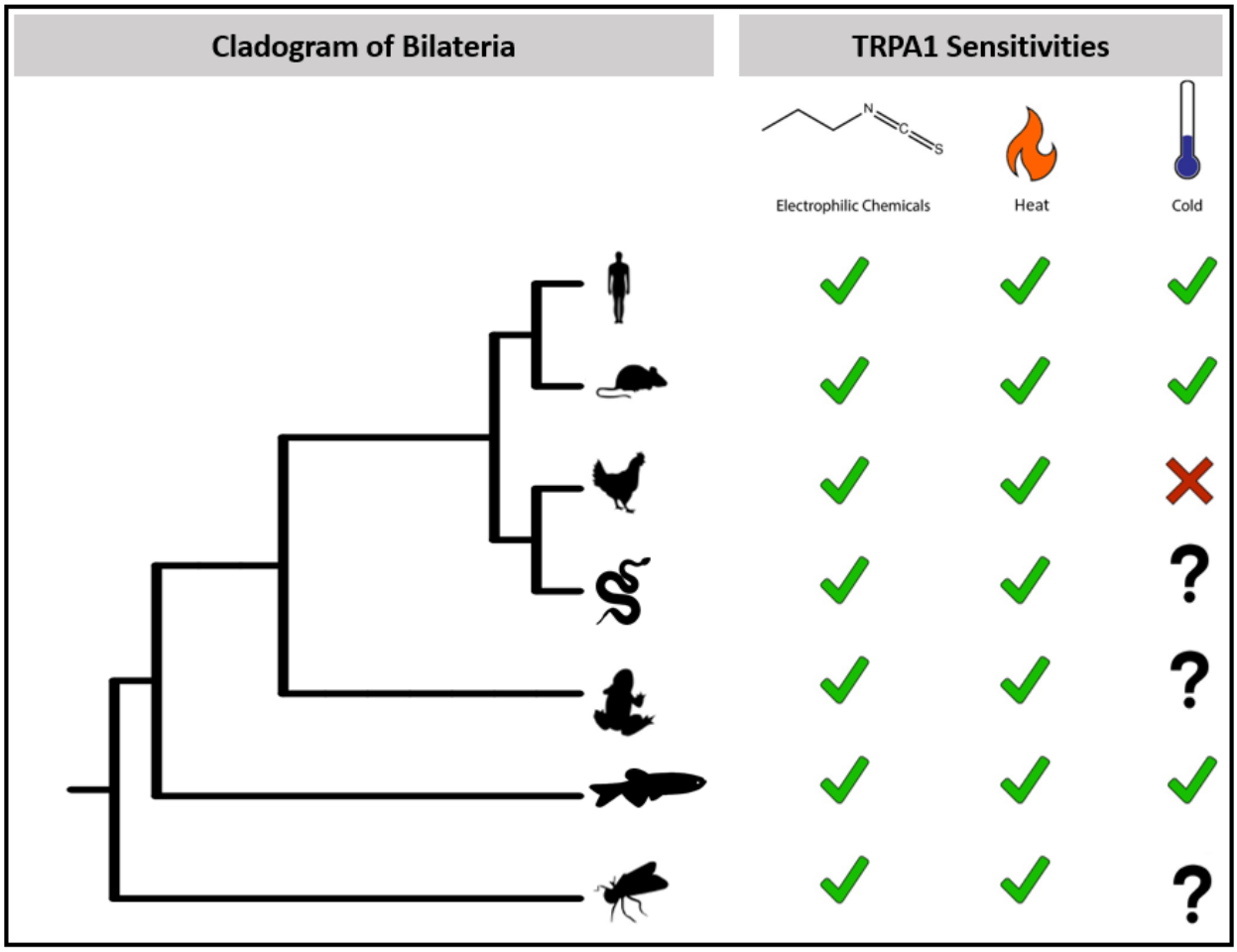

Figure 2-1. *Cladogram of Bilaterian* TrpA1 *and known sensitivities*

TRPA1 channels across Bilateria share conserved sensitivities to electrophilic chemicals and to high heat. Mammalian TRPA1s are also cold sensitive, while those of birds and reptiles are not. Whether TRPA1s of amphibians, other deuterostomes, or protostomes are cold sensitive remains unknown.

2.2 Results

TrpA1 *is required for nocifensive response to cold*

To test the hypothesis that *TrpA1* is required for cold nociception, we applied global noxious cold stimulus via cold plate assay (Turner et al., 2016). Larvae were placed onto a thin, room temperature arena and allowed to acclimate. Once locomotion resumed, the plate was transferred to a Peltier device pre-chilled to 8°C, a temperature noxious to fly larvae (**Fig. 2-2**) (Takeuchi et al., 2008). To assess a *TrpA1* requirement, we tested homozygous whole-animal mutant 3rd instar larvae using three distinct null alleles (Gu et al., 2019; Kwon et al., 2008; Zhong et al., 2012). Noxious cold evoked a CT response in significantly fewer *TrpA1* mutant larvae than in either wild-type or genetic control larvae (**Fig. 2-3A,B**), demonstrating that *TrpA1* is required for larval cold nociception. We observed no deficiency in either the magnitude of CT (**Fig 2-3C**) or latency to CT (**Fig. 2-3D**) in those mutant larvae that did CT. Because *TrpA1* mutations do not impact CT magnitude or latency, we focused the remainder of our cold plate behavioral analyses solely on percent contractors.

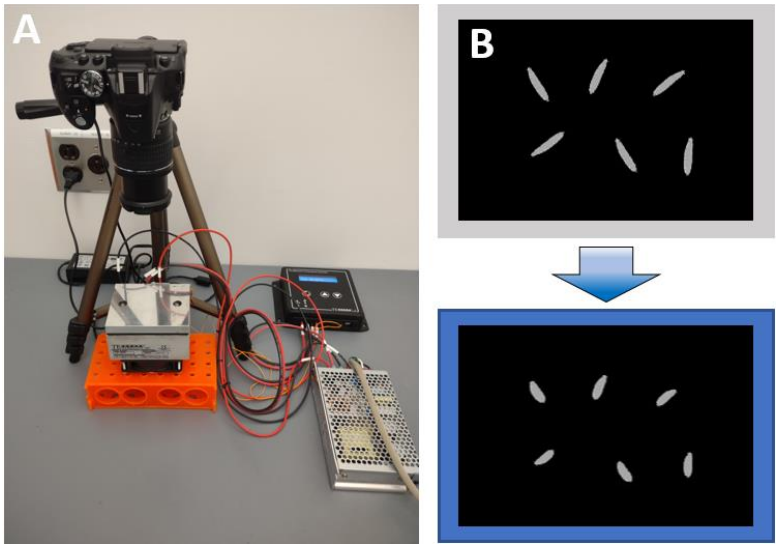

Figure 2-2*. Cold plate behavioral assay*

(**A**) Cold plate behavioral assay and recording apparatus. Larvae are placed onto a thin, room temperature aluminum arena and allowed to acclimate. The arena is then transferred to a Peltier plate pre-chilled to 8°C and recorded from above. (**B**) Cartoon illustration of freely locomoting larvae (above) and larvae exhibiting stereotyped nocifensive CT response to cold stimulus (below).

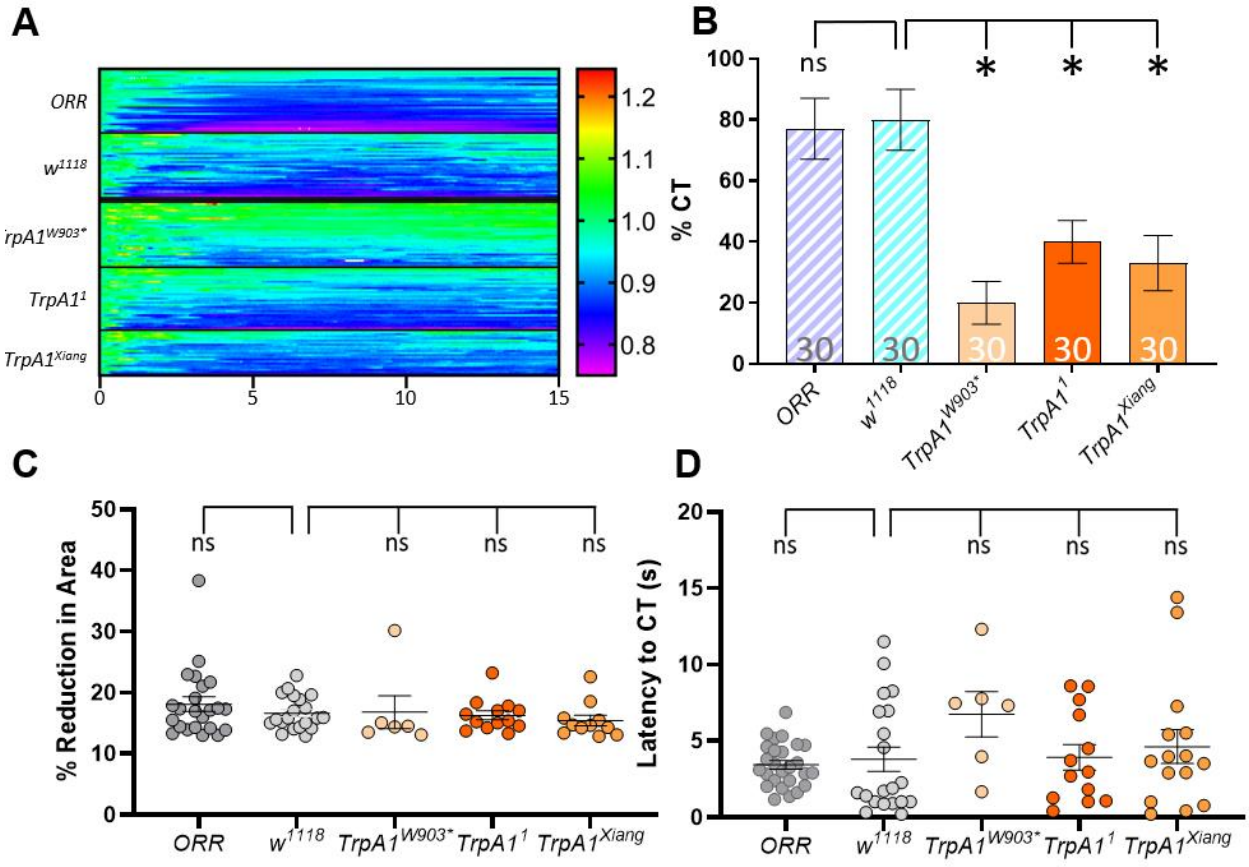

Figure 2-3*. Larvae mutant for* TrpA1 *display impaired cold-evoked CT*

TrpA1 mutant larvae exhibit significant deficiency in CT response to noxious cold. (**A**) Heatmap representing change in larval area during the first 15 s of cold stimulus. (**B**) Fewer larvae homozygous for *TrpA1* null mutant alleles CT relative to genetic controls (w^{1118}). CT behavior is comparable between genetic controls and wild-type (*ORR*) larvae. * indicates $p<0.0167$; ns indicates $p>0.05$. Of those larvae that did CT, mutants display no defect in (**C**) magnitude of CT or in (**D**) latency to CT, relative to controls. ns indicates $p>0.05$

There is no sex-specific requirement for* TrpA1*-mediated cold-evoked CT

A growing body of literature indicates that nociceptive pathways can differ between females and males within species (Shansky & Murphy, 2021). Of particular relevance, cold plate and acetone paw withdrawal assays in *TrpA1* mutant mice reveal deficiencies in female, but not male, nocifensive responses, suggesting distinct functional requirements for the channel between the sexes (Kwan et al., 2006). To assess whether there may exist a sex-specific role for *TrpA1* in larval cold nociception, we evaluated the CT responses of larvae of each sex, again using the cold plate assay. Similar percentages of female and male larvae contracted in response to noxious cold, both in genetic control and in *TrpA1* mutant genotypes (**Fig. 2-4**). For this reason, the remainder of our larval experiments were performed on mixed sex population sample groups.

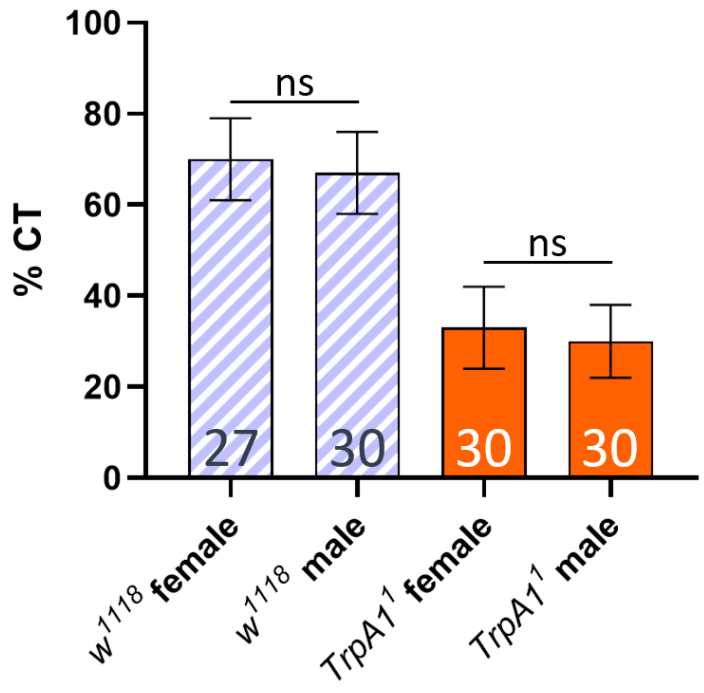

Figure 2-4. TrpA1*-mediated CT is independent of sex in* Drosophila *larvae.*

Female and male larvae exhibit similar cold-evoked nocifensive CT in both genetic controls and *TrpA1* mutant conditions. ns indicates $p>0.05$.

TrpA1 ***mediates cold nociception via CIII neurons***

CIII sensory neurons are the primary cold nociceptors in *Drosophila* larvae, and *TrpA1* is enriched in these neurons (Turner et al., 2016). Because *TrpA1* functions as a noxious cold detector in the nociceptors of other species (del Camino et al., 2010; Cordero-Morales et al., 2011; Kobayashi et al., 2005) (Story et al., 2003), we hypothesized that this receptor channel acts CIII-specifically to mediate cold nociception in *Drosophila* larvae. However, *TrpA1* is also expressed in distinct larval sensory neuronal populations, where it mediates chemical and high heat nociception, as well as in the central nervous system (Himmel et al., 2019; J. Luo et al., 2017; Zhong et al., 2012). To determine whether the *Drosophila TrpA1* requirement for proper CT behavior is CIII-specific, we utilized the *GAL4-UAS* binary expression system to knock down expression of *TrpA1* only in CIII neurons. Upon exposure to a noxious cold plate, significantly fewer CT responders were observed in the CIII knockdown condition than in genetic controls, to a degree resembling that of whole animal mutants (**Fig. 2-5A**), supporting our hypothesis of a CIII-specific role for *TrpA1* in cold nociception.

CIII-mediated cold nociception exhibits an isoform-A-specific functional requirement

Drosophila expresses at least five distinct *TrpA1* isoforms (A-E), which exhibit distinct expression patterns. Isoforms C, D, and E, are expressed in Class IV (CIV) md neurons, where they mediate responses to high heat, mechanical, and chemical nociception (Gu et al., 2019; Himmel et al., 2019; J. Luo et al., 2017; Viswanath et al., 2003). In larvae, isoforms A and B previously have been studied only in regard to their roles in central nervous system (J. Luo et al.,

2017). Given that, in other sensory modalities, *TrpA1* exhibits isoform-specific requirements, we hypothesized that this would be the case also with CIII-mediated cold nociception.

To determine the *TrpA1* isoform requirements for cold nociception, we utilized isoform-specific mutant alleles (Gu et al., 2019; J. Luo et al., 2017), which revealed significantly impaired CT in larvae mutant for isoform A, indicating that it is required in CIII neurons for proper nocifensive response (**Fig. 2-5B**). Mutations to isoforms B, C, and D did not significantly reduce CT behavior. Isoform E is expressed only in CIV polymodal nociceptors, which do not contribute to cold nociception (Gu et al., 2019), for which reason isoform E mutants were not tested. We next asked whether CIII-specific reintroduction of isoform A into whole-animal mutants was sufficient to rescue CT behavior. CIII-specific expression of *TrpA1-A*^{Kadowaki} (CIII>*TrpA1-A*^{Kadowaki}) in larvae led to a significant rescue of CT behavior upon noxious cold exposure relative to whole animal mutant and genetic controls (**Fig. 2-5C**), consistent with our hypothesis that isoform A acts via CIII neurons to mediate cold nocifensive behavior.

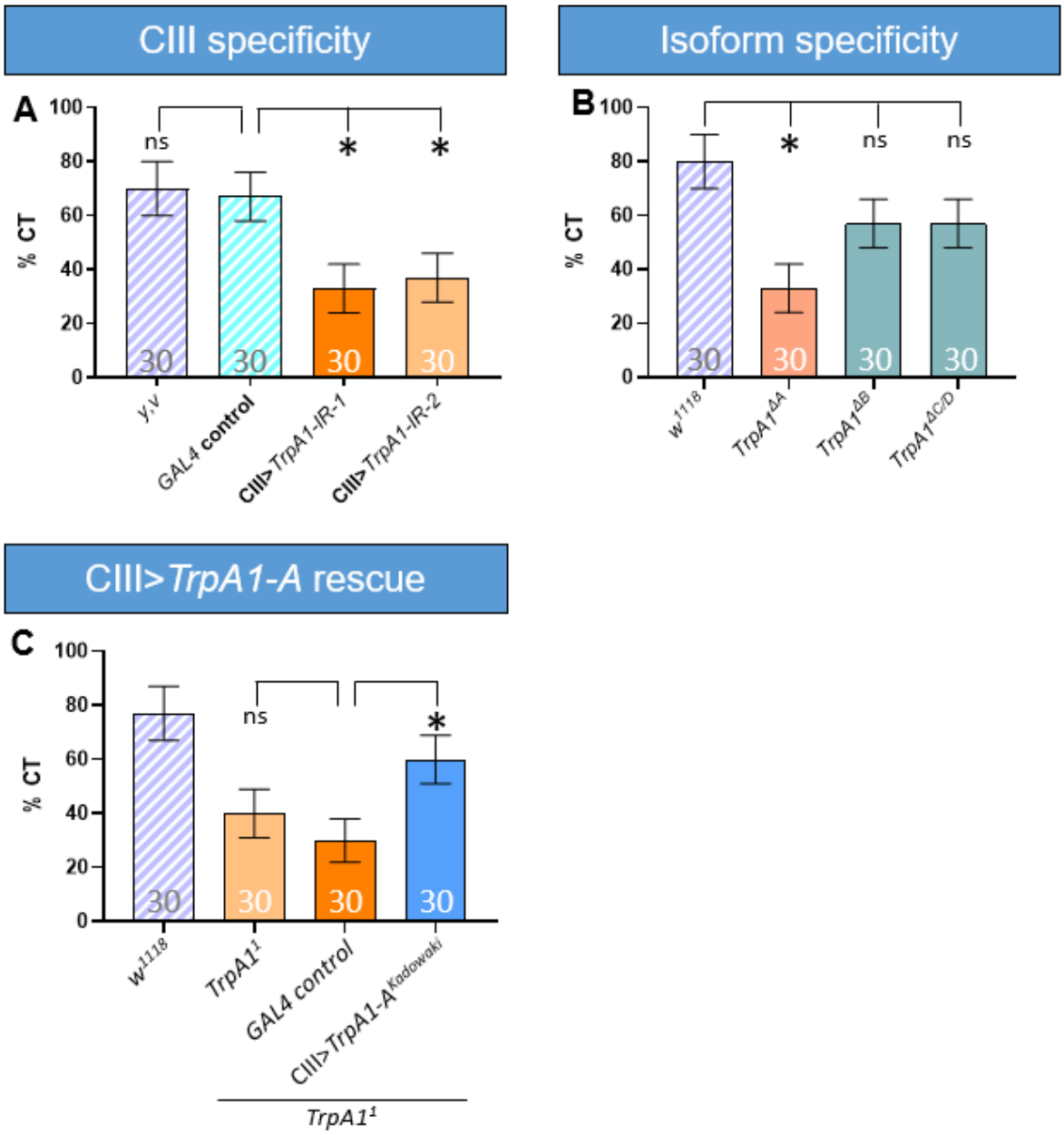

Figure 2-5. *Cold-evoked CT is mediated by* TrpA1 *isoform A functioning in CIII nociceptors.*

TrpA1 isoform A mediates cold-evoked CT via CIII nociceptors. (**A**) CIII-specific knockdown of *TrpA1* expression resulted in significantly fewer larvae contracting relative to controls. The first column (*y,v*) is the genetic background control for *IR* genotypes. * represents $p<0.0167$; ns represents $p>0.05$ (**B**) Among homozygous isoform-specific mutants, only larvae mutant for isoform A display significant impairment in CT response to noxious cold. * represents $p<0.0167$; ns represents $p>0.05$. (**C**) CIII-specific expression of *TrpA1* isoform A is sufficient to rescue CT behavioral response in whole-animal *TrpA1* mutant larvae. * represents $p<0.025$; ns represents $p>0.05$.

TrpA1 *ARD is functionally required for proper nocifensive CT response*

All TRPA1 orthologs are characterized by a long intracellular, N-terminal Ankyrin repeat domain (ARD), the functional roles of which remain incompletely understood (Fowler & Montell, 2013; Gaudet, 2008). The ARD is critical for high heat detection in both *Drosophila* and mammals (Cordero-Morales et al., 2011; Jabba et al., 2014). However, whether this requirement holds for cold detection is unclear (Clapham & Miller, 2011; Moparthi et al., 2014). To determine whether the ARD is required for noxious cold detection in *Drosophila*, we developed structure-function mutants possessing an inducible *UAS-TRPA1-A* element lacking the ARD (*UAS-TRPA1-A*$^{\Delta ARD}$). Misexpression of this transgene resulted in a severe reduction in CT nocifensive response to cold (**Fig. 2-6B**). Immunohistochemical staining demonstrates that the ARD mutant channel is trafficked to the dendrites, suggesting that the behavioral phenotype is not the result of mislocalization of the channel (**Fig. 2-6A**,**A'**).

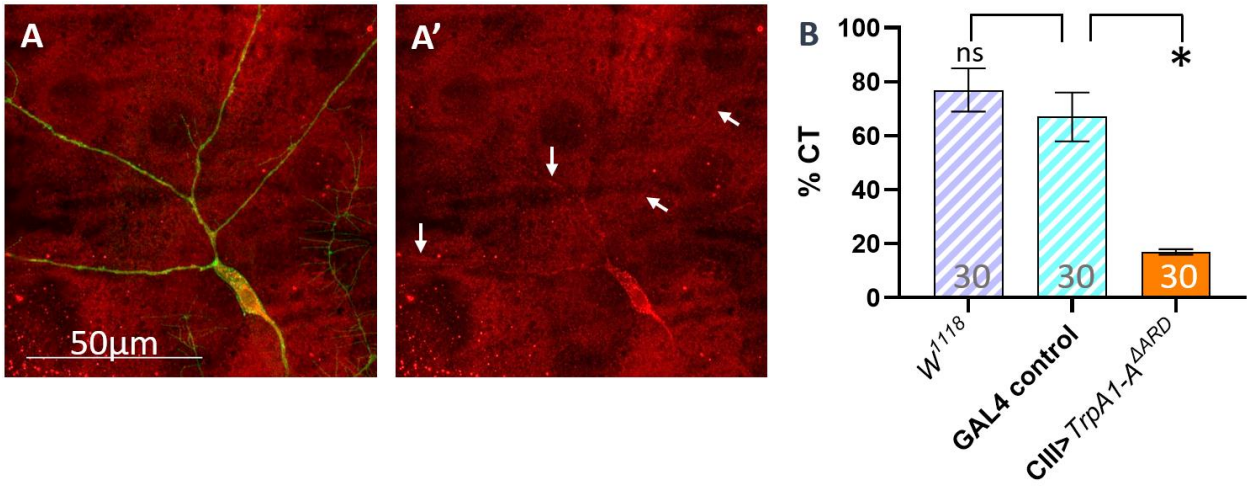

***Figure 2-6**. The ARD is required for CT response.*

TRPA1's N-terminal ARD is required for cold-evoked CT. (**A**) IHC labeling against membrane-expressed mCD8::GFP (green) and *TrpA1-A*$^{\Delta ARD}$-FLAG (red) in a representative CIII neuron. (**A'**) Magenta channel showing *TrpA1*$^{\Delta ARD}$ expression in CIII dendrites; arrows indicate magenta signal against background for clarity. (**B**) Larvae in which *TrpA1-A*$^{\Delta ARD}$ is expressed CIII-specifically CT significantly less than controls. * represents $p<0.025$; ns represents $p>0.05$

TrpA1 ***is not required for the establishment of dendritic receptive field***

The above results align with our hypothesis that *TrpA1* is required specifically for CIII-mediated cold detection. However, reduced CT might also result from a diminished receptive field, from abnormalities in neuronal connectivity, from a generalized locomotive impairment, or from reduced excitability of CIII neurons. We therefore tested all of these alternative hypotheses. To evaluate whether *TrpA1* expression is required for the proper establishment of dendritic field, we examined CIII morphology in *TrpA1* knockdown animals. Here, we saw no significant difference in either total dendritic length or number of branches in CIII>*TrpA1-IR* larvae relative to controls, suggesting that *TrpA1* is not required for CIII morphogenesis (**Fig. 2-7A-D**).

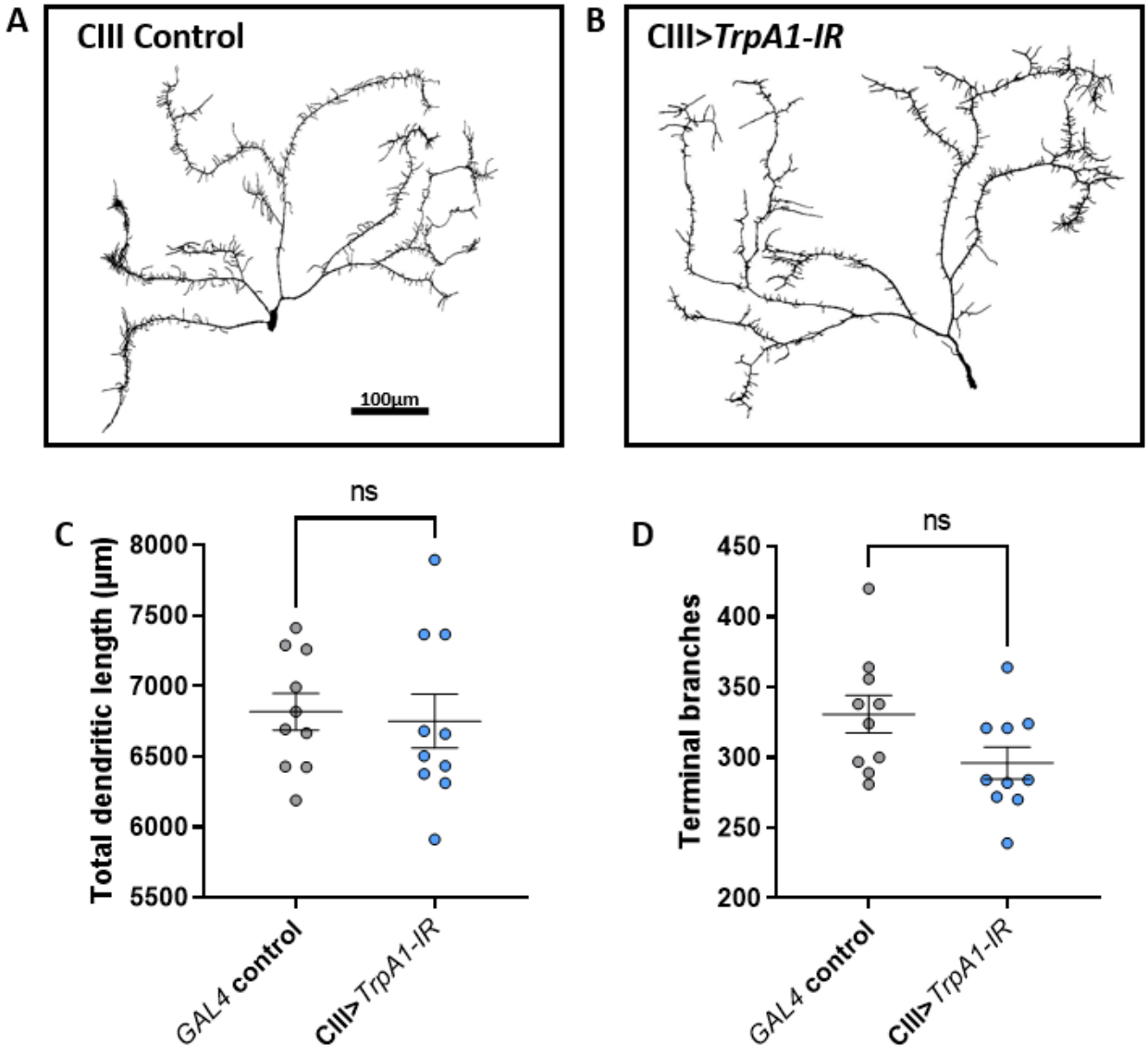

Figure 2-7. TrpA1 *is not required for CIII morphogenesis.*

TrpA1 is not required for establishment of CIII dendritic field. (**A**,**B**) Representative images of CIII neurons in (**A**) genetic controls and (**B**) CIII-specific *TrpA1* knockdown. (**C**) There is no significant reduction in total dendritic length of *TrpA1-IR* CIII neurons. Error bars represent SEM. ns indicates $p>0.05$. (**D**) There is no significant reduction in number of terminal branches in *TrpA1-IR* CIII neurons relative to those of controls. Error bars represent SEM. ns indicates $p>0.05$.

TrpA1 ***is dispensable for the transmission of CIII neuronal activity***

TRPA1 is highly permeable to Ca^{2+}, which is itself critical to the establishment of proper neuronal circuitry (Mattson, 1992). To distinguish between a general role of *TrpA1* in neuronal excitability versus a requirement for transmission of CIII neuronal activity to downstream interneurons, we sought to test whether optogenetically activating CIII neurons knocked down for *TrpA1* could still lead to cold-evoked CT behavior. If *TrpA1* were required for downstream conveyance of CIII activity, versus a specific role in thermosensory transduction, we predicted that optogenetic activation of CIII neurons in a *TrpA1-IR* background would fail to produce CT behavior upon blue light activation. Optogenetically activated CIII neurons expressing the modified channelrhodopsin ChETA in both CIII>*TrpA1-IR* larvae and in genetic controls revealed no significant difference in CT behavior in *TrpA1* knockdown larvae relative to controls (**Fig. 2-8A-D**), indicating that disruption of *TrpA1* expression in CIII neurons does not impair the development of functional connections with their downstream partners in the cold nociceptive circuit, but instead plays a role in cold sensory transduction.

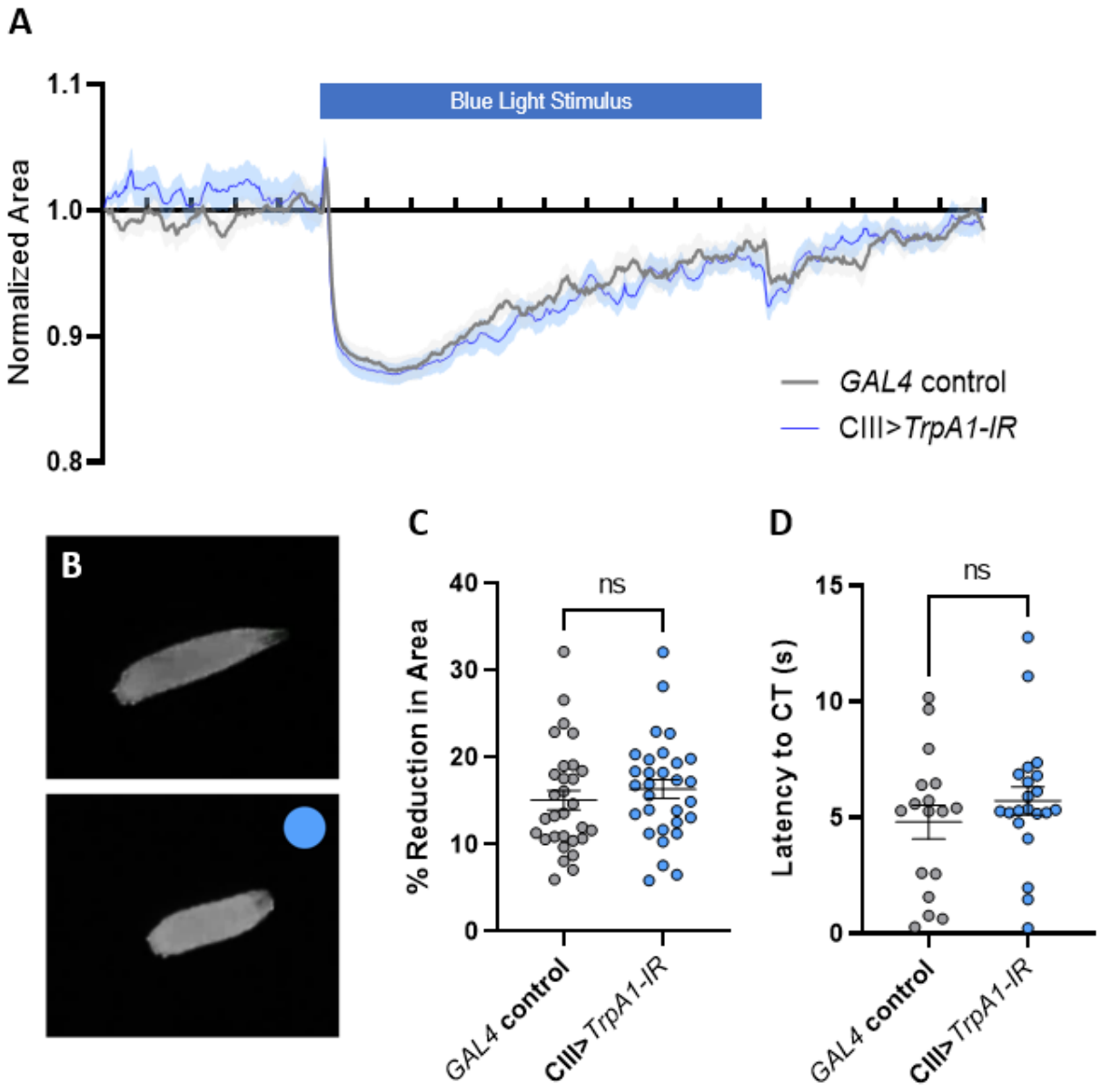

Figure 2-8. *TrpA1* is not required for the transmission of CIII neuronal activity

TrpA1 knockdown larvae exhibit no deficiency in CT behavior in response to optogenetic activation. (**A**) Average percent change in larval area (as measured from above) in *ChETA*-expressing *TrpA1-IR* and control larvae. Each tick on the x-axis = 1 second. Data reported are the mean ± SEM. (**B**) Representative images of a larvae locomoting before blue light stimulation (above) and contracting in response to blue light (bottom). (**C**) There is no significant difference in magnitude of CT between CIII>*TrpA1* larvae and controls. Error bars represent SEM. ns indicates $p>0.05$. (**D**) There is no significant difference in latency to CT between experimental and genetic control larvae. Error bars represent SEM. ns indicates $p>0.05$.

TrpA1 ***mutants exhibit no locomotive defects***

Diminished CT response in *TrpA1* mutants might be due to a generalized decrease in mobility in these larvae. To examine this possibility, we quantified their rates of locomotion and found no significant difference between mutants and genetic controls (**Fig. 2-9**).

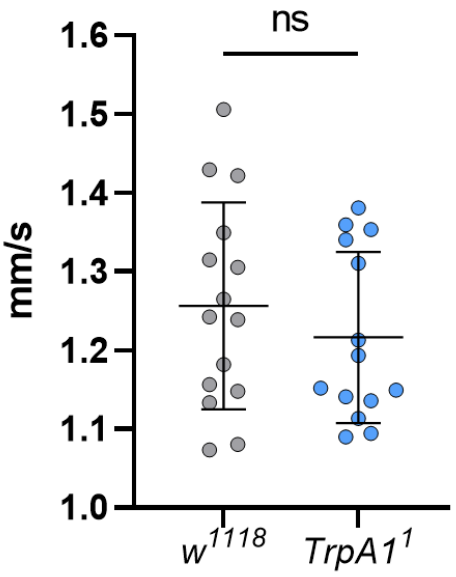

Figure 2-9. TrpA1 *mutation does not impair larval locomotion*

TrpA1 mutant larvae on a 2% agar arena locomote at rates equivalent to those of wild-type larvae. Error bars represent SEM. ns indicates $p>0.05$.

TrpA1 ***is selectively required for cold detection in polymodal CIII neurons***

CIII neurons are polymodal, responding not only to noxious cold stimuli, but also to innocuous mechanical stimuli (Tsubouchi et al., 2012; Yan et al., 2013). Prior analyses of thermoTRP channels including Pkd2, NompC and Trpm revealed that each of these genes is required in CIII neurons for gentle touch mechanosensation (Tsubouchi et al., 2012; Turner et al.,

2016; Yan et al., 2013); however, it is unknown if *TrpA1* is required for gentle touch mechanosensation. Polymodality is a common feature of TRP channels, including *TrpA1* (Babcock et al., 2011; Himmel & Cox, 2020; Kang et al., 2010; Kim et al., 2010; Neely et al., 2011; Xiang et al., 2010). We thus sought to examine whether *TrpA1* functions in a polymodal capacity in CIII neurons or if *TrpA1* is required in these neurons specifically for noxious cold detection. We performed a gentle touch assay as previously described (Turner et al., 2016), and observed no significant difference in response to innocuous mechanical stimulation between *TrpA1* mutant larvae and genetic controls, indicative of a modality-specific role of *TrpA1* in CIII neurons is noxious cold detection (**Fig. 2-10**).

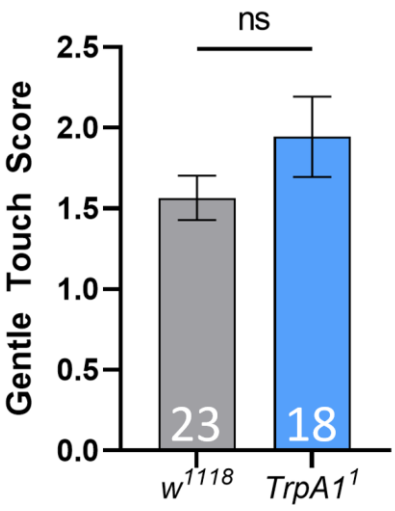

Figure 2-10. TrpA1 *is selectively required for cold detection in polymodal CIII neurons*

TrpA1 mutant larvae exhibit normal CIII-mediated mechanosensation, revealing a modality-specific role for *TrpA1* in cold nociception. Data are represented as the mean gentle touch score across larvae within each group ± SEM. (See **2.3 Materials and Methods**.) ns indicates p>0.05.

TrpA1 ***expression is sufficient to confer cold sensitivity***

To test the hypothesis that *TrpA1* can confer cold sensitivity, we overexpressed *TrpA1* isoform A in CIII neurons. We observed no significant increase in the percentage of CT responders in this condition relative to controls (**Fig. 2-11A**). As a very large proportion of animals normally CT at 8°C, we considered the possibility that there may be a ceiling effect beyond which CT will not occur in a greater proportion of larvae at this temperature (8°C). We then examined whether *TrpA1* can confer cold sensitivity onto otherwise non-cold-sensing cells. To accomplish this, we ectopically expressed *TrpA1-A* in adult GMR18B07 interneurons and evaluated the animals' behavior using a modified proboscis extension response (PER) assay (**Fig. 2-11B**,**C**) (Gordon & Scott, 2009; Schwarz et al., 2017). $GAL4^{GMR18B07}$ drives expression in interneurons which activate motor neurons 8 and 9 of the adult proboscis, resulting in a raising of the rostrum and extension of the labella (collectively, PER). We therefore predicted that noxious cold would not evoke a PER in wild-type animals, but that ectopic *TrpA1-A* expression would sensitize these neurons to cold. Significantly more animals expressing *TrpA1-A* in GMR18B07 interneurons exhibited PER than controls when challenged with a noxious cold stimulus (**Fig. 2-11D**), suggesting that *TrpA1-A* is sufficient to confer cold sensitivity onto otherwise non-cold-sensing cells. *Drosophila TrpA1* has been employed as a thermogenetic tool for the selective activation of targeted neuronal populations by heat (Bernstein et al., 2012; Hamada et al., 2008). Likewise, rat *Trpm8* has been used to thermogenetically activate neurons using cold stimulus (Peabody et al., 2009). Our results suggest that cold temperatures might likewise be used in conjunction with *Drosophila TrpA1* for thermogenetic applications.

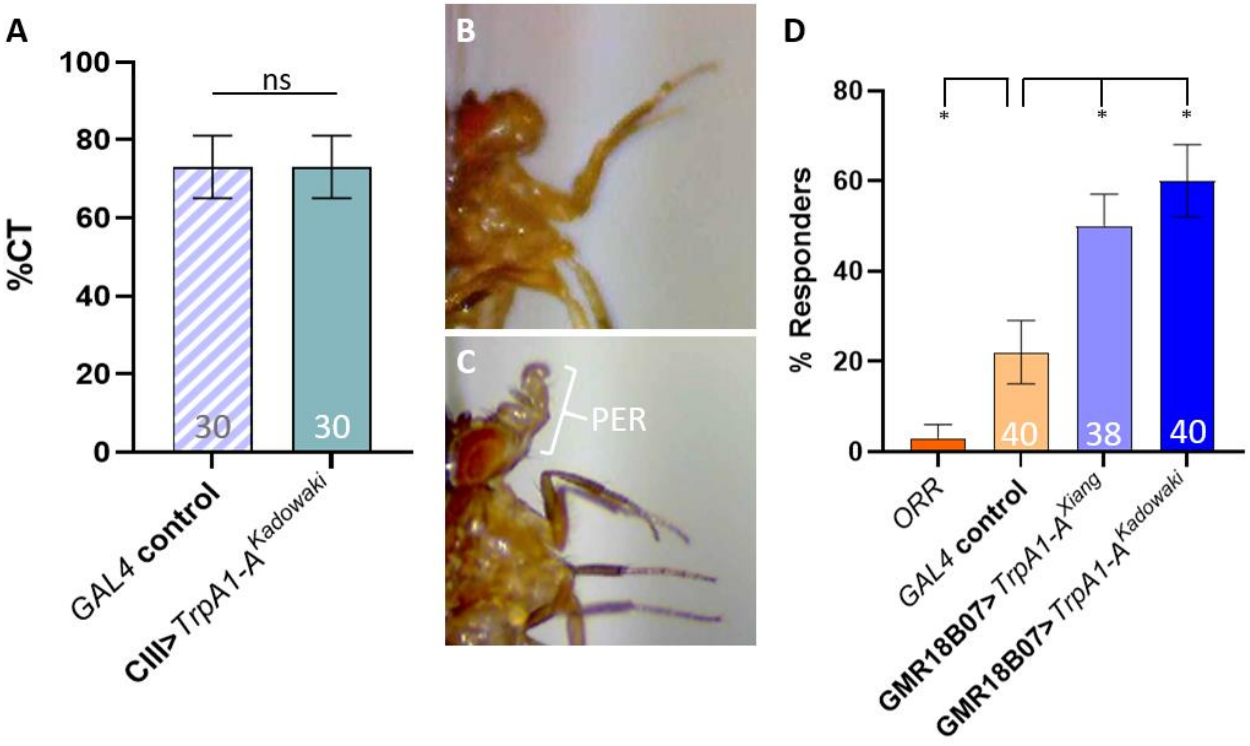

Figure 2-11. TrpA1 *is sufficient to sensitize non-cold-sensing cells to noxious cold.*

TrpA1 sensitizes neurons to cold. (**A**) CIII-specific *TrpA1* overexpression does not significantly increase CT behavior relative to controls. Error bars represent SEP. ns indicates p>0.05. (**B**) Representative *GAL4* control fly. (**C**) Representative *GMR18B07>TrpA1-A* fly mounted to cold plate exhibiting PER. (**D**) *TrpA1* overexpression confers sensitivity on non-cold-sensing interneurons in adult flies. *ORR* n=33. Error bars represent SEP. * indicates p<0.0167.

Genetically encoded Ca^{2+} sensors fail to reveal a role for* TrpA1 *in mediating cold-evoked Ca^{2+} activity

TrpA1 is a non-selective cation channel that is permeable to Ca^{2+} (Stueber et al., 2017; Wang et al., 2008). We therefore predicted that knocking down *TrpA1* in CIII neurons would result in decreased intracellular Ca^{2+} transients upon cold exposure. To test this, we employed the genetically encoded Ca^{2+} sensor *GCaMP6m* in genetic controls and in CIII>*TrpA1* knockdown larvae, subjecting them to a cold ramp regimen as depicted in (**Fig. 2-12A**). *GCaMP* fluorescence failed to evidence a decrease in intracellular Ca^{2+} transients in response to noxious cold stimulus

across the experimental regimen (**Fig. 2-12B**,**C**). Considering reports that *TrpA1* encodes rate of temperature change (Luo et al., 2017), we considered that impairment of Ca^{2+} transients might be observed only during the period of cooling (**Fig. 2-12D**) or in periods immediately following onset of cooling stimulus (**Fig. 2-12E**,**F**). However, again, *GCaMP* fluorescence did not reveal significant differences between *TrpA1-IR* CIII neurons and those of controls.

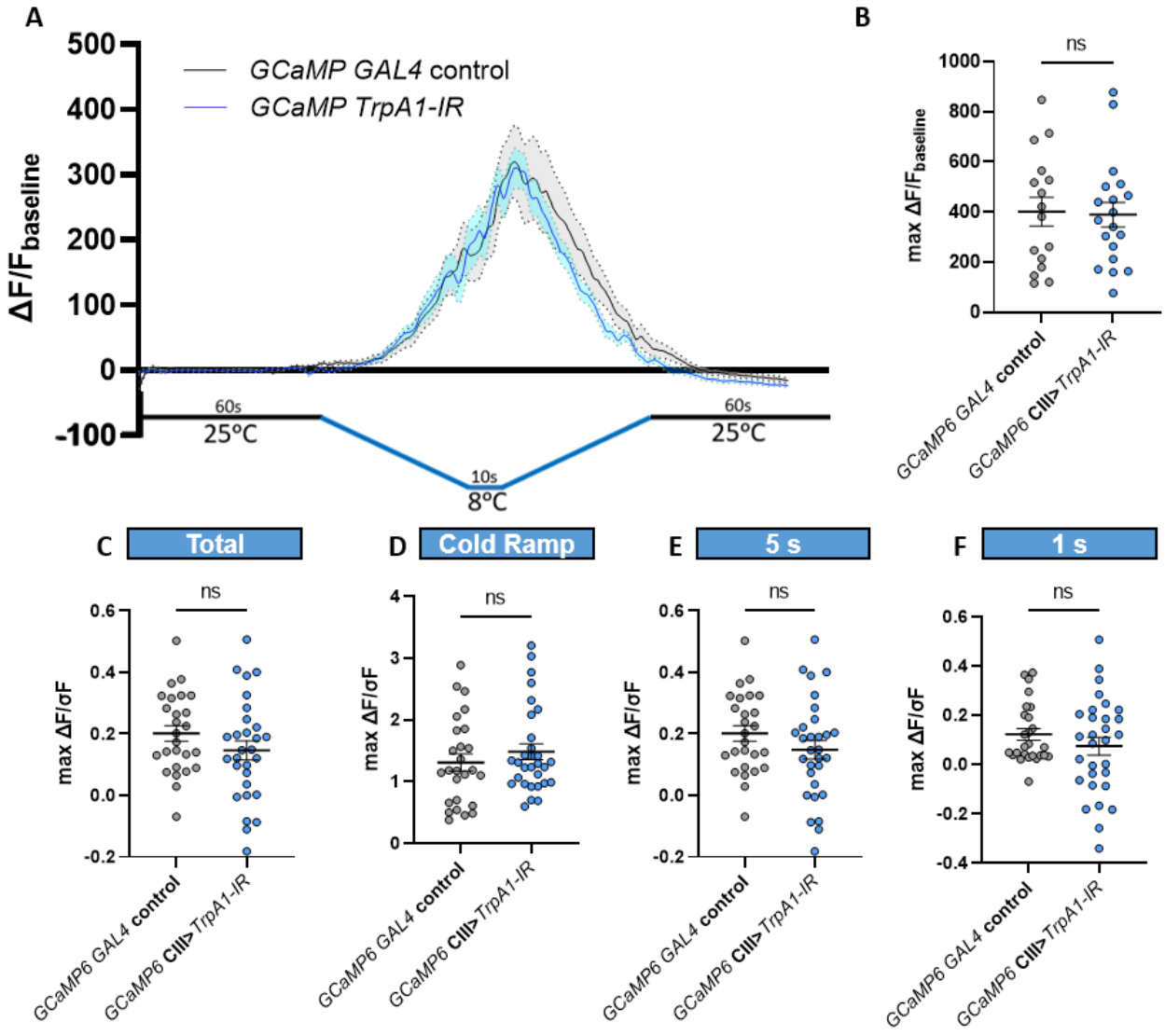

Figure 2-12. GCaMP6m *does not reveal impaired calcium transients in CIII neurons under* TrpA1-IR *conditions.*

CIII>*GCaMP6m* does not reveal differences in Ca^{2+} transients in CIII neurons of *TrpA1-IR* larvae relative to those of controls. (**A**) Averaged *GCaMP6* responses in CIII neurons subjected to noxious (8°C) cold cycle, depicted underneath. (**B**) Maximal $\Delta F/F_{baseline}$ throughout experiment does not reveal significant a difference between *TrpA1-IR* and controls. ns indicates p.0.05. (**C-F**) Maximal $\Delta F/\sigma F$ does not reveal a significant difference between *TrpA1-IR* and control CIII neurons when examined (**C**) across the entire experimental regimen, (**D**) during the cold ramp from 25°C to 8°C, during the initial 5 s of cooling, nor during the initial 1 s of cooling. ns indicates p>0.05.

The practical limit to the rate of temperature change for our cooling apparatus is 20°C/minute. We considered that TRPA1 may respond only to more rapid cooling. We therefore utilized the genetically encoded Ca^{2+} integrator *CaMPARI* in conjunction with the cold plate assay. *CaMPARI* fluoresces green in the absence of Ca^{2+}. When bound by Ca^{2+} in the presence of a photoconverting light, *CaMPARI* photoconverts to red fluorescence. We predicted that CIII>*TrpA1-IR* neurons would exhibit less photoconversion than control CIIIs. However, *CaMPARI* failed to reveal a diminishment in Ca^{2+}-evoked photoconversion (**Fig. 2-13**), possibly because TRPA1 is only one of several Ca^{2+}-permeant TRP channels mediating cold-evoked CIII activity (Turner et al., 2016). As intracellular Ca^{2+} is a proxy for neuronal firing, we sought instead to directly measure CIII electrical activity.

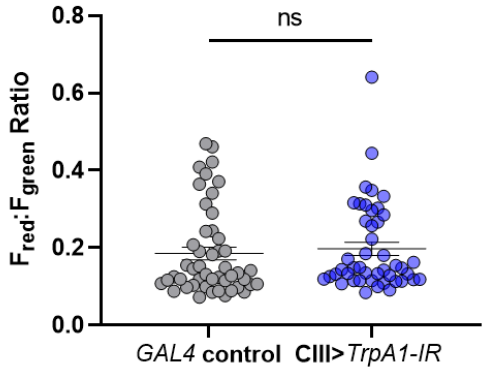

Figure 2-13*. The Ca^{2+} integrator* CaMPARI *does not reveal altered intracellular Ca^{2+} in* TrpA1-IR *CIII neurons*

CaMPARI does not reveal diminished Ca^{2+} activity in CIII>*TrpA1-IR* neurons in response to rapid, noxious cold insult. ns indicates p>0.05.

TrpA1 ***is required for cold-evoked neuronal firing***

We subsequently conducted extracellular electrophysiological recordings of control and *TrpA1* knockdown CIII neurons in order to determine how *TrpA1* may contribute to cold-evoked electrical responses. We applied cold-temperature stimulation of three different magnitudes (**Fig. 2-13**), the lowest temperature being 10°C—the practical limit of our chilling apparatus. In the *GAL4* control animals, all CIII neurons responded to 10°C and 15°C stimuli (n=21), and 90% responded to 20°C stimuli (n=19 of 21). All of the CIII neurons expressing *TrpA1-IR* showed spiking responses to the 10°C stimulus (n=18), 82.3% responded to the 15°C stimulus (n=14 of 17), and 73.3% responded to the 20°C stimulus (n=11 of 15). The average spiking rate during the 60 second period of stimulation of the neurons expressing *TrpA1-IR* was significantly lower than in the *GAL4* control neurons. (See **Fig. 2-14** legend.)

TrpA1 knockdown also altered spike firing patterns; the frequency of clustered spike discharges or bursting activity was greatly reduced in CIII neurons expressing *TrpA1-IR*. In *GAL4* controls, approximately half of the CIII neurons showed bursting activity early in the 10°C stimulus (n=13 of 21, **Fig. 2-14A**), which decreased as the intensity of the stimulus was reduced (15°C, n=11 of 21; 20°C, n=7 of 21). The bursting activity was most prominent immediately after the onset of the 10°C stimulus when the bursting spikes showed the highest frequency. The bursting spikes' frequency declined when the temperature reached a steady-state, which was then replaced by tonic spiking activity (**Fig. 2-14C**, bar graph for 10°C stimuli). Both the maximum frequency of bursting spikes and the magnitude of their steep decay became less pronounced as the stimulus temperature became milder (**Fig. 2-14C**). In cells expressing *TrpA1-IR*, bursting spikes were largely absent (**Fig. 2-14B**); only 16.7% (n=3 of 18) of the neurons exhibited bursting

spikes during the 10°C stimulation, 23.5% (n=4 of 17) cells at 15°C stimulation, and in 13.3% (n=2 of 15) of cells at 20°C stimulation (**Fig. 2-14D**). There was a marked decrease in the frequency of maximal bursting spikes, but not of tonic spikes, during the stimulation period in cells expressing *TrpA1-IR* compared to *GAL4* controls at 10°C and 15°C stimuli (**Fig. 2-14C,D**; **Fig. 2-15A-C**).

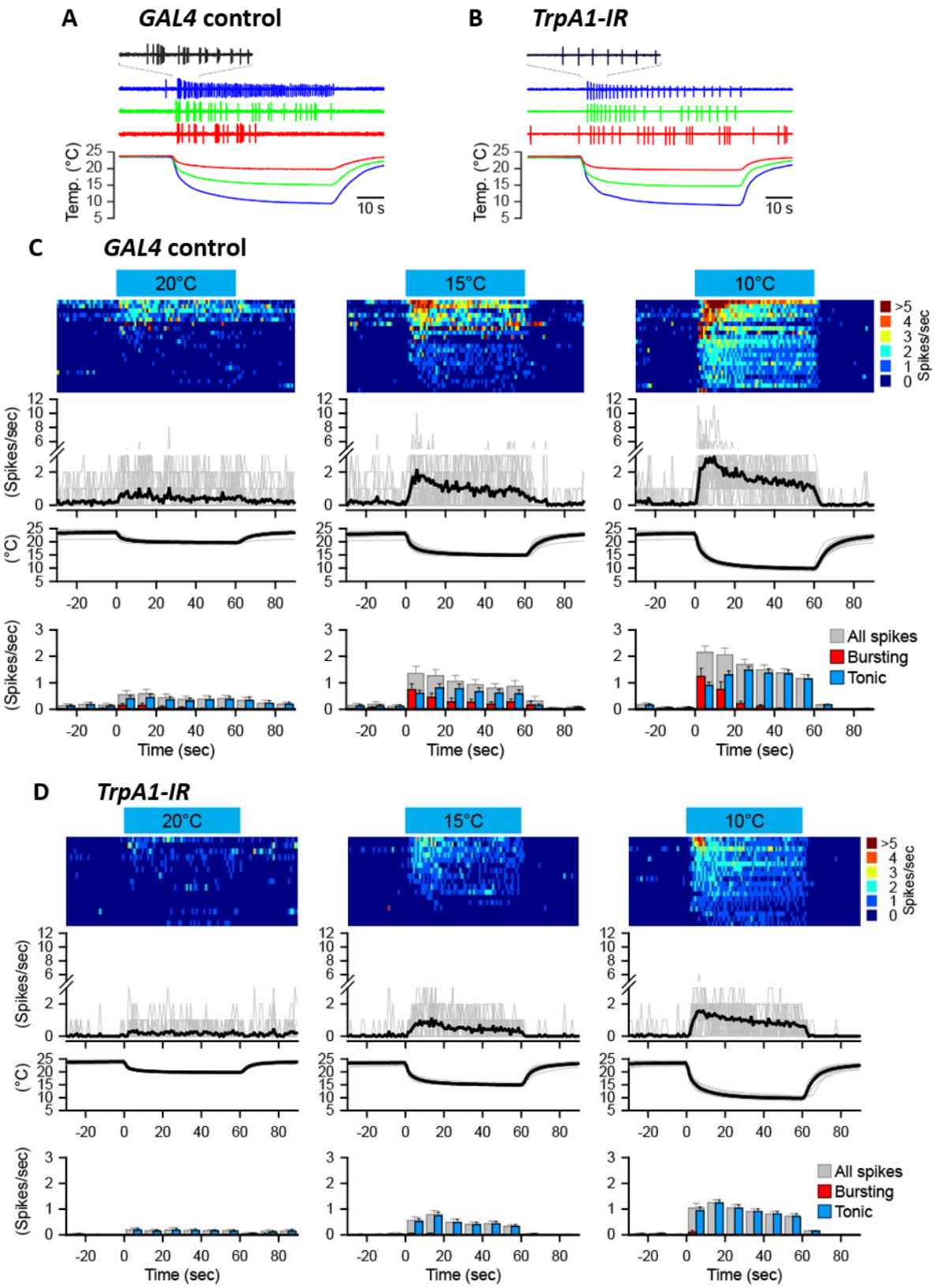

Figure 2-14. Electrophysiological recordings reveal impaired electrical activity in *TrpA1-IR* CIII

neurons relative to that of controls

(A,B) Representative spiking activities of CIII neurons of (**A**) *GAL4* control and (**B**) *TrpA1-IR* in response to corresponding low temperature stimuli of three different magnitudes (red, 20°C; green, 15°C; blue, 10°C). Enlarged traces show spiking responses during the 10 s period immediately after the onset of 10°C stimulus. **(C,D)** The panels show, from top to bottom, a heat map representation of spiking rate, plots of averaged (black) and individual (grey) spiking rates (spikes/s) against time, average traces of temperature (°C), and bar graphs showing averaged frequencies (spikes/s, mean ± SEM) of intra-burst spikes (red bars) and tonic spikes (cyan bars) in every 10-s bin. The grey bar behind red and blue bars indicates the averaged frequency of all spikes in each bin. n=21 for *GAL4* controls (**C**), n=18 for *TrpA1-IR* (**D**).

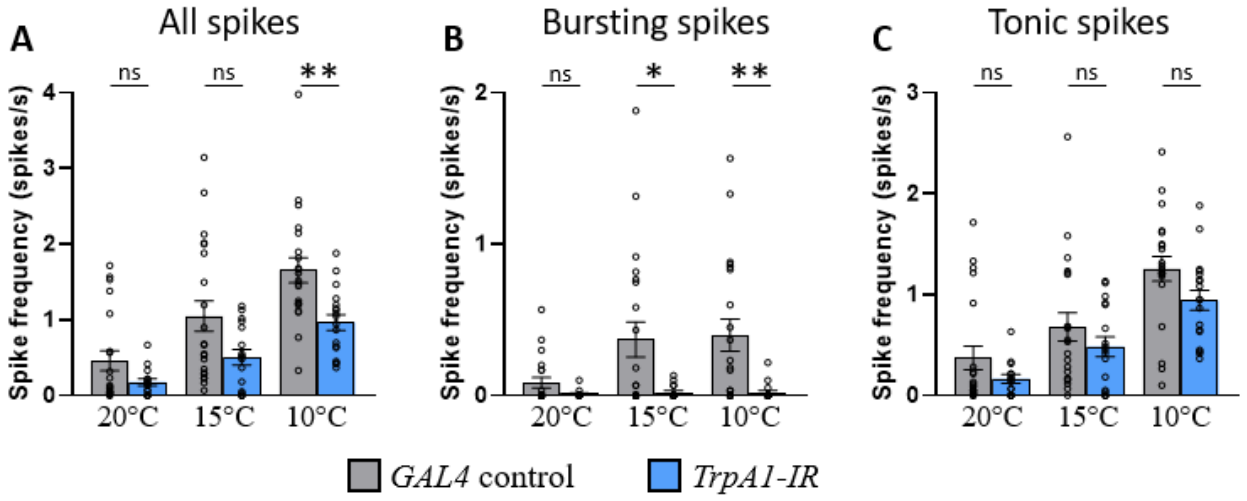

Figure 2-15. TrpA1 *is required for neuronal bursting*

Bursting activity is significantly impaired in *TrpA1* knockdown CIII neurons. The bar graphs show the comparison of averages of (**A**) all spike frequencies, (**B**) bursting spike frequency, and (**C**) tonic spike frequency between *GAL4* control (grey) and *TrpA1-IR* (blue) during the 60-s cold temperature stimulation. There was a significant difference between the two groups in all spike frequency produced by 10°C stimulation (**A**, Mann-Whitney *U* test: $p = 0.001$; n=21 for *GAL4* controls, n=18 for *TrpA1-IR*), and in burst spike frequency by 10°C and 15°C stimulation (**B**, Mann-Whitney *U* test: 10°C, $p < 0.001$ n=21 for *GAL4* control, n=18 for *TrpA1-IR*; 15°C, $p < 0.02$, n=21 for *GAL4* control, n=17 *TrpA1-IR*).

Human TrpA1 *functionally rescues CT behavior in mutant* Drosophila *larvae*

TrpA1 sensitivities to stimuli of non-thermal modalities are broadly conserved across taxa. The ability to respond to noxious high heat is also widespread. However, noxious cold sensitivity has previously been reported only in the mammalian lineage. Among birds and reptiles, where *TrpA1* is sensitive to high heat, cold temperature responses have not been investigated (reviewed in Laursen et al., 2015). That *Drosophila TrpA1* mediates both high heat and cold nociception suggests the possibility that noxious cold sensing is ancestrally derived and may be shared across numerous animal phyla. To test our hypothesis that *TrpA1* cold sensitivity is ancestral, we developed a phylogenetic tree with representatives including *TrpA1* from a species closely related to *Drosophila melanogaster* (that of *Drosophila mojavensis*) and *TrpA1* from the much more distantly related *Homo sapiens*. Also represented are other *TrpA* genes, including the honeybee TRPA channel *Apis mellifera* $TrpA^{AmHs}$, which belongs to the *water witch* family that is thought to have diverged from the *TrpA1* lineage prior to the evolution of thermal sensitivity (Kohno et al., 2010; Himmel et al. 2019). (**Fig. 2-16A**). We performed cross-species rescue experiments using *UAS-TrpA1* constructs from both *Drosophila mojavensis* and human, as well as *UAS-*$TrpA^{AmHs}$. We observed that larvae misexpressing either *TrpA1* transgene exhibited rescue of cold-evoked nocifensive CT, while rescue was not detected in larvae ectopically expressing $TrpA^{AmHs}$. (**Fig 2-16B**). The functional compensation by both closely and distantly related *TrpA1*s, and the lack of rescue by $TrpA^{AmHs}$, suggest that the cold responsiveness of this channel is ancestrally derived.

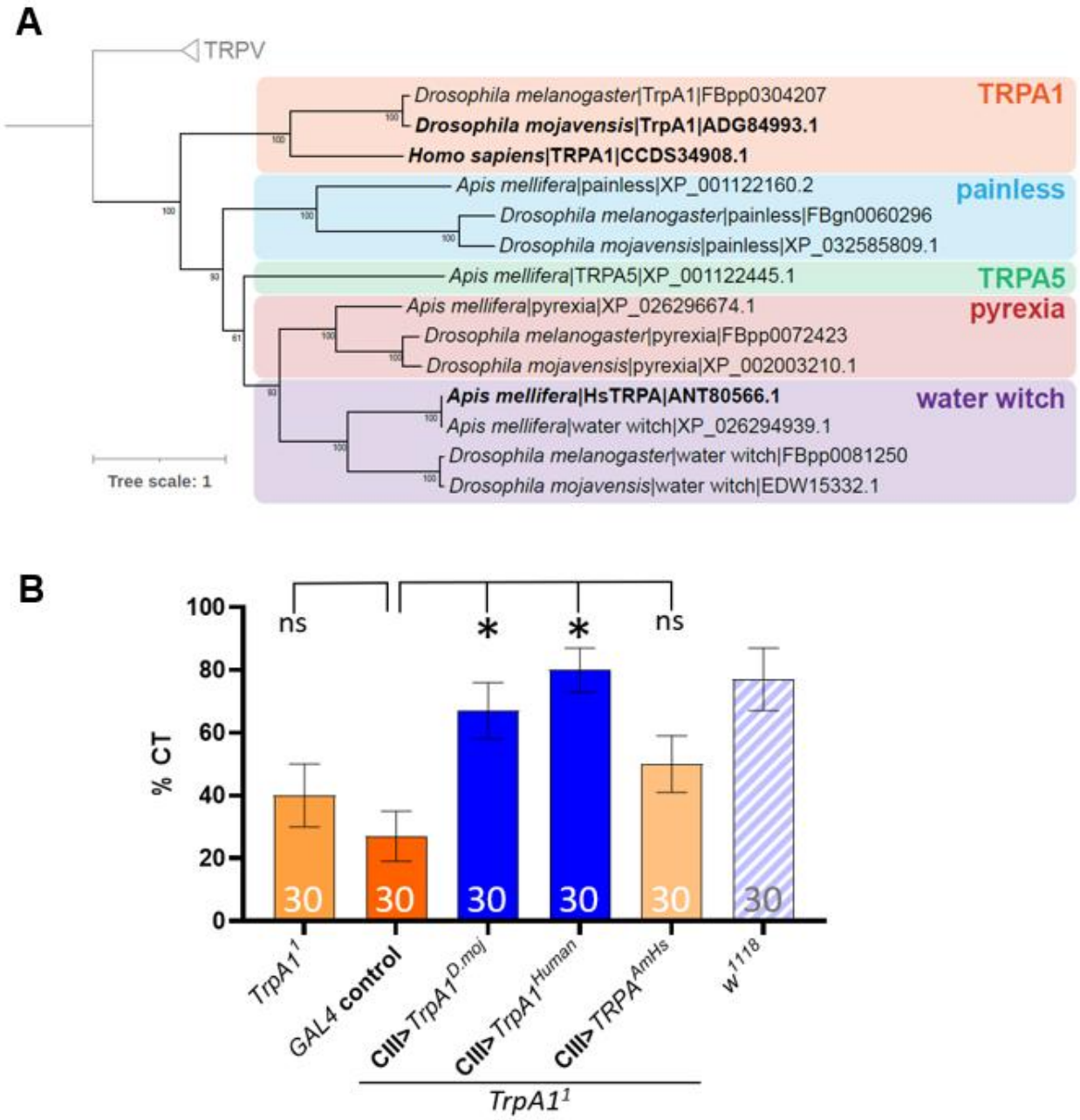

Figure 2-16. *Larval CT behavior is rescued by CIII>specific expression of* TrpA1s *of other species*

(**A**) Phylogenetic tree of *TrpAs* of representative species. The ancestor of *TrpA*^{AmHs} diverged from the *TrpA* lineage prior to the evolution of *TrpA1* thermosensitivity. (**B**) CIII-specific misexpression of either the closely related *Drosophila mojavensis* or the distantly related *Homo sapiens TrpA1* orthologs results in significantly increased CT behavior relative to mutant controls, revealing cross-species rescue. * indicates $p \leq 0.001$. ns indicates $p=0.0315$, not statistically significant after Bonferroni correction for multiple comparisons.

Gustatory Receptor* Gr28b *functions in CIII neurons to mediate noxious cold nociception

Recently, our group has found via transcriptomic analyses that the gustatory receptor (GR) *Gr28b* is expressed in CIII neurons (data not shown; Turner et al., 2016). Although the GRs were originally identified by their role in insect gustatory sensation, recent investigations have revealed roles for the GRs in the detection of stimuli of different modalities, such as noxious high heat (Ni et al., 2013; Simões et al., 2021). We hypothesized therefore that *Gr28b* functions in CIII neurons to mediate cold nociception, and evaluated this hypothesis using the cold plate assay. Homozygous *Gr28b* mutant larvae evinced a requirement for this gene in cold nociception (**Fig. 2-17A**), and *Gr28b-IR* knockdown experiments confirmed a CIII-specificity for this requirement (**Fig. 2-17B**). In the adult fly antenna, *Gr28b* can functionally substitute for *TrpA1* as a heat detector (Ni et al., 2013), so we performed cold plate experiments in animals transheterozygously mutant for both of these genes to evaluate whether they genetically interact in their roles as noxious cold sensors. Both *TrpA1* and *Gr28b* are haplo-insufficient for cold-evoked CT behavior, with significantly fewer heterozygous mutants responding to cold than genetic controls. Transheterozygous *Gr28b/+; TrpA1/+* mutant larvae also displayed impaired CT responses, comparable to those of monogenic heterozygote mutants, suggesting that these genes act in parallel in CIII-mediated cold nociception (**Fig. 2-17C**).

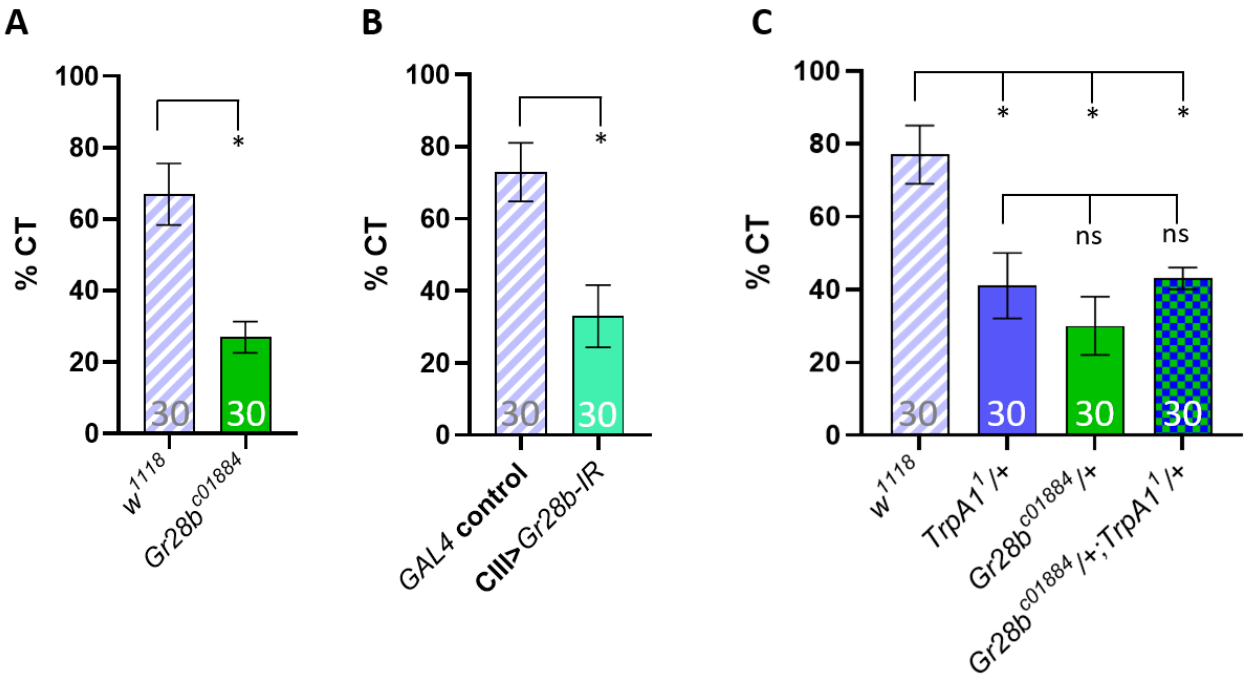

Figure 2-17. Gr28b *is required in CIII neurons for cold nociception.*

Gr28b is required in CIII neurons for cold nociception. (**A**) Significantly fewer *Gr28b* homozygous mutant larvae CT in response to noxious cold, relative to controls. * represents $p<0.05$. (**B**) CIII-specific knockdown of *Gr28b* results in significantly fewer larvae CTing relative to controls. * represents $p<0.05$. (**C**) *TrpA1* and *Gr28b* are both haplo-insufficient, as larvae heterozygously mutant for either gene exhibit significantly diminished CT behavior. Larvae transheterozygously mutant for both genes exhibit CT behavior comparable to that of both *TrpA1* and *Gr28b* heterozygous mutants.

2.3 Materials and Methods

2.3.1 Drosophila *strains and husbandry*

All *Drosophila melanogaster* stocks were maintained at 25°C under a 12:12 light:dark cycle on cornmeal agar food. Genetic crosses were incubated under the same conditions. All experiments, excepting the proboscis extension response experiment (**Fig. 2-7**), utilized unsexed 3rd instar larvae. The PER experiment utilized adult flies of both sexes, aged 7-12 days post eclosure. All *GAL4* driver lines were outcrossed either to *UAS-Luciferase-IR* or to $TrpA1^{1}$ to serve as genetic controls. Luciferase is not produced by *Drosophila melanogaster*, and *luc-IR* has no predicted off target effects; it therefore serves as a *UAS* control. *TrpA1* outcrosses are indicated in the relevant Figures. Publicly available transgenic strains were obtained from Bloomington Drosophila Stock Center (BDRC) and include: *OregonR*, w^{1118}, $TrpA1^{1}$, y^{1},v^{1}, *UAS-Luciferase-IR*, *UAS-TrpA1-IR*, $TrpA1^{\Delta C/D}$, $GAL4^{GM418B07}$, *UAS-TrpA1-*$A^{Kadowaki}$, *UAS-GCaMP6m*, $TrpA1^{1}$,*UAS-TrpA1*$^{D.\ mojavensis}$, $TrpA1^{1}$,*UAS-TrpA*^{AmHs}, $Gr28b^{c01884}$, and *UAS-Gr28b-IR*. Catalog numbers are provided in **Table 3**. $TRPA1^{1}$,*UAS-TrpA1*^{Human} was a gift of Dr. M Gallio (Arenas et al., 2017). $GAL4^{19\text{-}12}$ was a gift of Dr. YN Jan (Xiang et al., 2010). $TrpA1^{W903*}$ was a gift of Dr. WD Tracey (Zhong et al., 2012). $TrpA1^{Xiang}$, $TrpA1^{\Delta A}$, $TrpA1^{\Delta B}$, and *UAS-TrpA1-*A^{Xiang} were gifts of Dr. Y Xiang (Gu et al., 2019). *ppkGAL4,ppkGAL80* was previously developed by this lab (E. P. R. Iyer et al., 2013), and is available upon request. *UAS-TrpA1-*$A^{\Delta ARD}$ and *UAS-CaMPARI;*$GAL4^{677}$ were developed for this study and are available upon request.

2.3.2 Cold plate behavioral assay

Global application of noxious cold stimulus was performed via cold plate assay as described in (Turner et al., 2016). Briefly, 3rd instar larvae were placed onto a thin, moistened, room temperature arena and allowed to acclimate. Once locomotion resumed, the arena was

transferred to a Peltier plate preset to 8°C. Behavior was video recorded from above at 30fps using a Nikon D5300. Videos were uncompressed using Video to Video conversion software (videotovideo.org). Larval area during the first 15s of stimulus exposure was then quantified in ImageJ. CT was defined as a reduction in surface area (as viewed from above) of >12.8%, as determined by the mean magnitude of CT in wild-type larvae minus 1.5 standard deviations. See (Turner et al., 2016). CT responders are reported as sample proportion ± standard error of proportion (SEP). Statistical analyses: one-tailed chi square test with Bonferroni correction.

2.3.3 Generation of UAS-TrpA1-A$^{\Delta ARD}$ transgenic fly line

We synthesized the *Drosophila melanogaster TrpA1* isoform A from which the entire Ankyrin repeat domain (ARD) was excised (GeneScript). The synthesized gene was FLAG-tagged at the N-terminus and subcloned into *pUAST-attB*. The transgenic strain was generated by ΦC31-mediated integration targeting 2L at cytological band 22A3 (*attP2-VK37*) (GenetiVision).

2.3.4 Immunofluorescent staining

Immunohistochemistry was performed as previously described in (Sulkowski et al., 2011). Primary antibodies used were mouse anti-FLAG (1:1000 dilution; Invitrogen) and chicken anti-GFP (1:1000 dilution; Abcam). Secondary antibodies were donkey anti-mouse 555 (1:200 dilution; Life Technologies) and donkey anti-chicken 488 (1:1000 dilution; Jackson Immunoresearch). Third instar larvae were fileted and mounted onto slides in Fluoromount Aqueous Mounding Media (Sigma). They were then imaged using a Zeiss LSM 780 confocal microscope with a 40X objective lens.

2.3.5 Live imaging confocal microscopy and morphometric analyses

Live imaging was performed as described in (E. P. R. Iyer et al., 2013; S. C. Iyer et al., 2013). Live 3rd instar larvae were placed onto a slide, immersed in 1:5 (v/v) diethyl

ether:halocarbon oil, immobilized under a coverslip, and imaged on a Zeiss LSM780 confocal microscope. Three dimensional z-stacks were obtained at 2μm intervals and at 1024 x 1024 resolution. Z-stacks were rendered into two-dimensional maximum intensity projections and exported as .jpeg files using Zen Blue software. Neurons were then skeletonized using Photoshope and a custom software, FlyBoys (available upon request). Morphometric quantification of dendritic arbors was performed in ImageJ as described in (E. P. R. Iyer et al., 2013; Schneider et al., 2012). Quantitative morphometric data were extracted using custom Python scripts. Total dendritic length ± standard error of the mean (SEM), as well as number of terminal branches ± SEM, are reported. Statistical analyses: unpaired t-test.

2.3.6 Optogenetics

Gal4$^{19\text{-}12}$,UAS-ChETA-YFP/UAS-TrpA1-IR and *Gal4$^{19\text{-}12}$,UAS-ChETA-YFP/UAS-Luc-IR* larvae were reared at 25°C in darkness on cornmeal agar + 1.5mM all-*trans*-retinal (ATR). Control larvae of the same genotypes were reared in darkness on food lacking ATR. Third instar larvae were subjected to 480nm light and their responses recorded at 30fps on a Nikon EOS Rebel T3i using Noldus EthoVision 12 software. Behavioral responses quantified in ImageJ and were evaluated as described above. Magnitude of CT (%) ± SEM, as well as latency to CT (s) ± SEM, are reported. Statistical analyses: unpaired t-test (**Fig. 2-6F**) and Mann-Whitney *U* (**Fig. 2-6G**).

2.3.7 Locomotion assay

The larval locomotion assay was performed as described in (Reddish et al., 2021). Briefly, 3rd instar larvae were placed onto a 2% agarose gel surface and allowed to acclimate for 5 minutes at room temperature. Larval locomotion was then recorded for 5 minutes at 30fps using a Nikon D5300. Videos were uncompressed using Video to Video conversion software (videotovideo.org) and locomotion was tracked using FIMTrack (Risse et al., 2017) and analyzed using custom

algorithms (available upon request). Data are presented as mm/s ± SEM. Statistical analysis: Mann-Whitney *U* test.

2.3.8 Mechanosensation assay

The gentle touch assay performed was slightly modified from (Kernan et al., 1994). Briefly, third instar larvae were placed onto a room temperature, 2% agar Petri plate and allowed to acclimate for one minute. Larval behavior was then recorded for 10 seconds without stimulation. Larvae were then stimulated by a single brush stroke in the thoracic (T1-T3) region, and their behavior was recorded for 5 seconds. Behavior was recorded using a Nikon D5300. Stereotyped larval responses to innocuous mechanical stimulation include: head withdrawal, turning of the animal's anterior end, single reverse peristaltic wave, and reverse locomotion. Each behavioral response was assigned a value of 1, and when a larva performed more than one behavior, these were summed. Results are reported as mean score ± SEM. Statistical analysis performed: Mann-Whitney *U* test.

2.3.9 **in vivo *calcium imaging* (GCaMP6m)**

CIII neuronal imaging was performed as described in (Turner et al., 2016). Briefly, *Gal4*$^{19\text{-}12}$*,UAS-mCD8::RFP* drove CIII expression of both *UAS-TrpA1-IR* and *UAS-GCaMP6m* in experimental larvae and *UAS-luc-IR* and *UAS-GCaMP6m* in controls. Third instar larvae were placed on a slide, immersed in water, and immobilized under a coverslip. The slide was then secured to a Linkam PE 120 Peltier plate affixed to a Zeiss LSM780 confocal microscope stage and the larvae left undisturbed at 25°C for 2 minutes. Larvae were then subjected to the following programmed temperature regime: 60s at 25°C, followed by a 20°C/s ramp to 8°C, constant 8°C stimulus for 10s, then a 20°C/s ramp to 25°C, after which larvae were recorded for another 60s at 25°C. The soma of a single ddaF CIII neuron of segments 4-6 (per larva) was recorded at 30fps

for the duration of the experiment. Videos were stabilized using ImageJ's StackReg plugin, and fluorescence quantified in ImageJ. Data are reported as change in fluorescence /standard deviation of baseline F ($\Delta F/\sigma F_{baseline}$) $\pm$ SEM as described previously in (Hamm et al., 2021; Wenzel & Hamm, 2021). Statistical analysis: Mann Whitney *U* test.

2.3.10 in vivo *Calcium Imaging* (CaMPARI)

GAL4^{677} was used to drive the expression of *UAS-CaMPARI* and either *UAS-Luc-IR* (*GAL4* control) or *UAS-TrpA1-IR.* Larvae were subjected to either no photoconverting light (PC) + no cold (8°C) stimulus, PC + no cold stimulus, no PC + cold stimulus, PC + no stimulus, or PC + stimulus. PC was delivered to live, 3rd instar larvae via a Zeiss AxioZoom V16 using filter settings described in (Fosque et al., 2015), at 84,000 lux for 20 s. Cold stimulus was delivered via a Peltier plate as described in ***2.3.2 Cold plate behavioral assay***, above. Larvae were imaged as described above in ***2.3.5 Live imaging confocal microscopy***. Fluorescence change was calculated as previously described (Fosque et al., 2015) using maximum intensity projections via Zeiss Zen Blue in order to calculate F_{red}/F_{green} ratio. Statistical analysis: Mann Whitney *U* test.

2.3.11 Proboscis Extension Response assay

Adult flies (3 to 12 days after eclosure; (Chung et al., 2017)) were placed individually into empty vials. Each fly was anesthetized under CO_2 and its wings removed using microdissection scissors and forceps as described in (Martin et al., 2018). A thin layer of Elmer's gel glue was applied to a coverslip and the animal was placed onto the glue dorsal side down. An additional drop of glue was place anterior to the fly's head and the fly was moved so that the head was directly over this drop. A paintbrush was used to lightly press and secure the fly, ensuring that the head and thorax were completely mounted to the coverslip. A brief enclosure in CO_2 following gluing was used to prevent excessive movement and ensure proper mounting.

Flies were acclimated in this position on the coverslip for at least 30 minutes (Neely et al., 2011). A Peltier plate set to either 8°C or 25°C was positioned vertically under a Zeiss Stemi 305 Microscope connected via Bluetooth to an iPad for recording. Each fly was placed individually onto the plate and recorded for at least ten seconds.

Videos were randomized and scored blind to genetic and temperature conditions. Behaviors were scored for 10s following stimulus onset. PER was defined minimally as the lifting of the rostrum. Data reported as percent responders ± SEP. Statistical analyses: one-tailed chi square with Bonferroni correction.

2.3.12 Electrophysiology

Third instar larvae were fileted and pinned in a Petri plate lined with Sylgard® 184 (Dow Corning). The dish was continuously superfused with gravity-dripped HL-3 saline. Cold stimulation to the neurons was carried out by direct application of chilled HL-3 saline. The saline was chilled to the desired temperature by passing it through an SC-20 in-line solution cooler (Warner Instruments) connected to the CL-100 temperature controller (Warner Instruments).

The spiking activity of CIII neurons was recorded extracellularly with a macropatch pipette with a tip diameter of 5–10 μm. The electrode was filled with HL-3 saline and connected via an Ag-AgCl wire to the headstage of a patch-clamp amplifier (MultiClamp 700A, Molecular Devices). By applying slight negative pressure, the cells were brought into close contact with the electrode tip. The amplified current signals were digitized at 10 kHz using a Micro1401 A/D converter (Cambridge Electronic Design) and acquired into a laptop computer running Windows 10 with Spike2 software (Cambridge Electronic Design). The spike frequency was counted by the Spike2 script, which was then introduced into MatLab (MathWorks, Inc) to create heatmaps. When three or more consecutive spikes with a spike interval of 0.2 s or less (*i.e.*, a frequency of 5

Hz or higher) occurred, they were considered bursts; all other spikes were defined as tonic spikes. The average frequencies of all spikes, bursting spikes, and tonic spikes were measured in each 10-second bin and plotted using SigmaPlot (Systat Software, Inc). Statistical analyses performed: Repeated measures ANOVA.

2.3.13 Phylogenetics

Sequences from *Drosophila melanogaster* (FlyBase), *Drosophila mojavensis* (NCBI), *Apis mellifera* (NCBI), and *Homo sapiens* (CCDS Database) were aligned via MAFFT using default settings. The phylogenetic tree was generated in IQ-TREE by the maximum likelihood approach, using an LG+F+I+G4 substitution model (as determined by ModelFinder). Branch support is indicated at branches and was calculated by ultrafast bootstrapping (UFboot, 2000 replicates). Trees were visualized using iTOL and Adobe Illustrator. Tips are labeled as species|protein|accession.

2.4 Collaborator Contributions

Erin Lottes performed immunohistochemistry experiments (**Fig. 2-6A**,**A'**). Atit Patel developed a customized FIMTrack pipeline for the quantification of larval locomotion (**Fig. 2-9**). He also developed a customized EthoVision protocol for performing optogenetic experiments (**Fig. 2-8**) and generated the CIII *GAL4* driver line used for *CaMPARI* experiments (**Fig. 2-13**). Anna Rader customized and performed the PER assay (**Fig. 2-11**), and assisted with associated data analysis. Dr. Akira Sakurai performed all electrophysiology experiments and developed graphical representations (**Figs. 2-14** and **2-15**). Dr. Nathaniel Himmel generated the phylogenetic tree (**Fig. 2-16A**) and analyzed data for the CIII>$TrpA^{AmHS}$ experiment (**Fig. 2-16B**). Dusty Moon

performed cold plate assays for CIII>*Gr28b-IR* (**Fig. 2-17B**). All other experiments, analyses, and statistics were performed by me.

3 INTRACELLULAR ROS EXACERBATES COLD-EVOKED CT BEHAVIOR

The functional requirement of a thermally insensitive TRPA1 channel for high heat nociception in *Drosophila* suggests that the activation of thermal nociceptors is not simply a function of temperature, but requires additional (or alternative) factors. Recent experiments have demonstrated that noxious heat causes the production of reactive oxygen species (ROS) in *Drosophila* larvae (Arenas et al., 2017). Electrophysiology experiments demonstrated that ROS is capable of activating not only *Drosophila* TRPA1, but also orthologs of distantly related species, including humans. Further, TRPA1 orthologs from distantly related species, such as humans, are capable of rescuing heat nocifensive behavior in *TRPA1* mutant *Drosophila*, suggesting a mechanism conserved across diverse lineages despite the fact that human TRPA1 has been linked to cold, not heat, nociception (Hoffstaetter et al., 2018). Therefore, elucidation of the evolutionarily conserved mechanisms underlying TRPA1-mediated cold nociception in *Drosophila* will help us to identify and further investigate the structures and functions that have been conserved across evolutionary history, and provide insight into human nociception.

Whether noxious cold generates ROS in *Drosophila* has not been explored. This study will test the hypothesis that intracellular ROS facilitates larval CT response to noxious cold. If this hypothesis is supported, it will provide a basis for further investigation into the possible causative role of noxious cold in generating ROS and the putative relationship between ROS and TRPA1 channel activation in CIII neurons.

3.1 Scientific Premise

CIII sensory neurons are polymodal, mediating responses both to innocuous mechanical and to noxious cold stimuli. However, the mechanism by which distinct behavioral responses are evoked by stimuli of different modalities has not been elucidated (Turner et al., 2016). Noxious

stimuli such as extreme cold are potentially damaging, and such cell damage often leads to the generation of ROS (Awad et al., 2013; Sun et al., 2016). Recent studies have demonstrated that ROS is capable of activating the TRPA1 orthologs of several species, including *Drosophila* TRPA1 expressed in CIV sensory neurons, which are responsible for mediating high heat nociception (Arenas et al., 2017; Miyake et al., 2016; Neely et al., 2011). Moreover, as TRPA1 functions in chemical, thermal, and mechanical nociception, ROS signaling may represent a unifying mechanism for indirect activation of TRPA1 thereby providing insight into how TRPA1 can respond to such a wide array of noxious sensory stimuli (Doerner et al., 2007; Viana, 2016).

3.2 Results

ROS facilitates larval CT response to noxious cold

ROS application has been shown experimentally to activate TRPA1 channels *in vitro* (Oda et al., 2016; Ogawa et al., 2016). Noxious high heat has been shown to increase ROS generation in adult flies, thereby exacerbating their nocifensive responses in a *TrpA1*-dependent manner (Arenas et al., 2017). Given that noxious cold stimuli are also known to increase ROS production in some cell types (Awad et al., 2013; Sun et al., 2016), we hypothesized that, in *Drosophila* CIII neurons, cold induces increased ROS formation, which in turn may directly or indirectly influence the opening of TRPA1 channels, thereby contributing to cold-evoked CT nocifensive behavior. To determine whether ROS influences the cold-evoked CT response in larvae, we overexpressed *dual oxidase* (*Duox*), which generates ROS independent of the mitochondria, in CIII neurons, and subjected larvae to the cold plate assay. Relative to controls, ectopic expression of *Duox* in CIII neurons resulted in a greater percentage of larvae contracting in response to noxious cold, suggesting that increased intracellular ROS leads to CIII sensitization to noxious cold stimuli (**Fig. 3-1**).

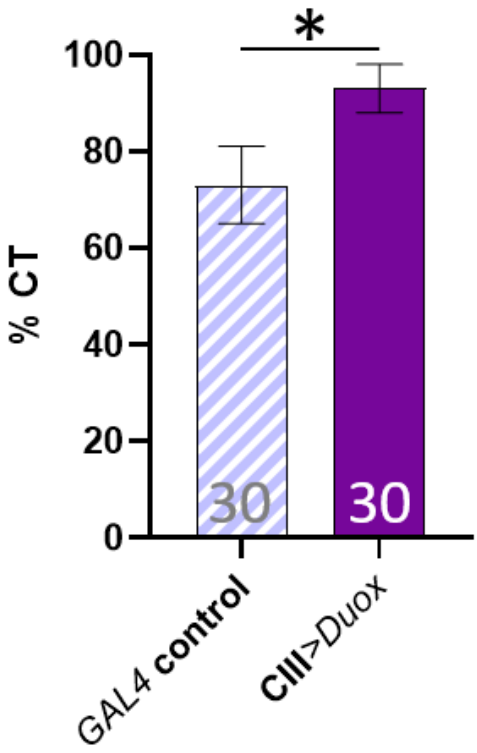

Figure 3-1. *Intracellular ROS facilitates CIII-mediated CT.*

CIII-specific misexpression of *Duox* significantly increases the proportion of animals that CT in response to noxious cold. * indicates p=0.0001.

Endogenous ROS mediates larval nocifensive CT behavior

We next sought to determine whether endogenous ROS is sufficient to mediate CT behavior. To evaluate this, we manipulated the levels of native ROS via two native ROS clearance enzymes, superoxide dismutase (SOD) and catalase (Cat). CIII-specific overexpression of *Cat* and *SOD* both resulted in significantly fewer larvae contracting (**Fig. 3-2A**), in support of our hypothesis. Intriguingly though, CIII>*Cat* and CIII>*SOD* knockdown larvae also exhibited either no significant impact or, in select RNAi lines, decreased CT relative to genetic controls (**Fig. 3-2B**). We hypothesize that long-term downregulation of these enzymes results in constitutive ROS

levels sufficiently high that they damage the neurons, thereby preventing proper function. (See also **4.1 Summary and Future Directions**.)

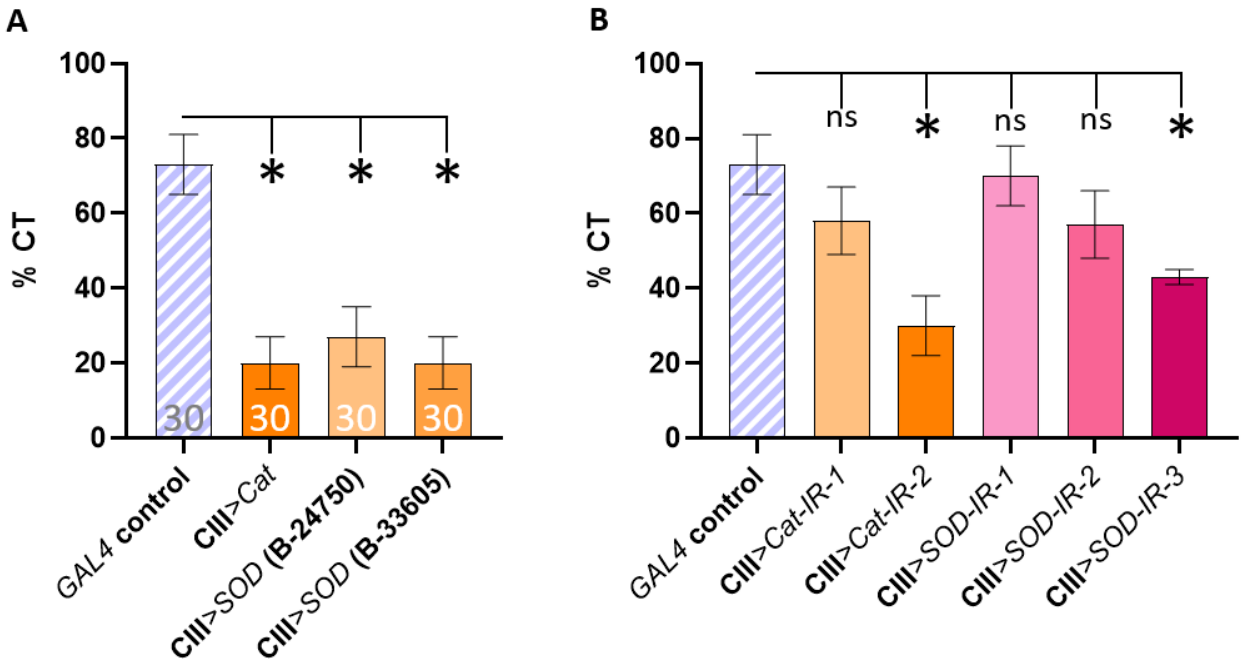

Figure 3-2. *Endogenous ROS mediates CT response.*

Endogenous ROS mediates noxious cold-evoked larval CT. (**A**) Significantly fewer CIII>*Cat* and CIII>*SOD* overexpression larvae contracted than did genetic controls. * represents $p \leq 0.0002$. (**B**) Significantly more CIII>*SOD-IR* larvae contracted than did genetic controls. * represents $p < 0.0001$. ns represents $p > 0.05$.

3.3 Materials and Methods

3.3.1 Drosophila *strains and husbandry*

All *Drosophila melanogaster* stocks were maintained at 25°C under a 12:12 light:dark cycle on cornmeal agar food. Genetic crosses were incubated under the same conditions. All experiments utilized unsexed 3rd instar larvae. *GAL4* driver lines were outcrossed to *UAS-Luciferase-IR* to serve as genetic controls. Luciferase is not produced by *Drosophila melanogaster*, and *luc-IR* has no predicted off target effects; it therefore serves as a *UAS* control. Publicly available transgenic strains were obtained from Bloomington Drosophila Stock Center (BDRC) and include: *UAS-Luciferase-IR*, *UAS-Duox*, *UAS-Cat*, *UAS-SOD*, *UAS-Cat-IR*, and *UAS-SOD-IR*. Catalog numbers are provided in **Table 3**. *GAL4*$^{19\text{-}12}$ was a gift of Dr. U Heberlein.

3.3.2 Cold plate behavioral assay

Global application of noxious cold stimulus was performed via cold plate assay as described in (Turner et al., 2016c). Briefly, 3rd instar larvae were placed onto a thin, moistened, room temperature arena and allowed to acclimate. Once locomotion resumed, the arena was transferred to a Peltier plate preset to 8°C. Behavior was video recorded from above at 30fps using a Nikon D5300. Videos were uncompressed using Video to Video conversion software (videotovideo.org). Larval area during the first 15s of stimulus exposure was then quantified in ImageJ. CT was defined as a reduction in surface area (as viewed from above) of >12.8%, as determined by the mean magnitude of CT in wild-type larvae minus 1.5 standard deviations. See (Turner et al., 2016). CT responders are reported as sample proportion $\pm$ standard error of proportion (SEP). Statistical analyses: one-tailed chi square test with Bonferroni correction.

3.4 Collaborator Contributions

Mariana Holguin-Lopez, Inara Jawed, Romee Maitra, and I performed cold plate assays for CIII>*Duox*, CIII>*Cat*, CIII>*SOD*, CIII>*Cat-IR*, and CIII>*SOD-IR* experiments. I performed all data processing and statistical analyses.

4 GENERAL DISCUSSION

TrpA1 participates in the detection of a remarkably diverse number of stimuli across multiple modalities. Although its function in chemical and mechanical nociception are broadly conserved, the evolution of *TrpA1* thermal nociception remains less clear. Historically, researchers have viewed noxious cold sensitivity as having evolved within the restricted lineages (Chen, 2015). The channel's role in high heat detection is better understood, having been investigated in organisms across a broad variety of taxa, from protostomes such as *Drosophila melanogaster* to vertebrates, including fish, amphibians, reptiles, and birds (Oda et al., 2016; Saito et al., 2014, 2016; Zhong et al., 2012). However, the view that *TrpA1* thermal sensitivity must be unidirectional has been challenged recently. Mice homozygously mutant for *TrpA1* demonstrate higher tolerance to noxious heat (Hoffmann et al., 2013). Computational studies have predicted that all thermoTRPs should be sensitive to both cold and high heat, regardless of their gating mechanism (Clapham & Miller, 2011). Zebrafish *TrpA1* has been shown to be sensitive to either temperature extreme in ectopic expression systems (Oda et al., 2016, 2018). Further, studies in lipid bilayers have demonstrated experimentally that mouse *TrpA1* is intrinsically sensitive to high heat and cold thermal stimuli, being activated by both *in silico*, although the exact physiological implications of this finding remain elusive (Moparthi et al., 2014a, 2016). Collectively, these data suggest that the biological role of *TrpA1* is more intricate than has been historically appreciated.

Drosophila melanogaster TrpA1 has been a subject of considerable investigation, and is known to mediate high heat nociception in larvae via the polymodal CIV sensory neurons (Chin & Tracey, 2017; Himmel et al., 2017; Tracey, 2017). CIV neurons are largely insensitive to cold, while the polymodal CIII sensory neurons function as the larva's primary cold nociceptor (Turner et al., 2016). Our group has previously documented that *TrpA1* is enriched in these cold

nociceptors (Turner et al., 2016), leading us to hypothesize that, in addition to its roles in CIV neurons, it also functions in the CIIIs to mediate cold nociception.

The studies herein have confirmed this hypothesis, demonstrating that global *TrpA1* mutants exhibit severe defects in cold-evoked CT behavior, indicating that *TrpA1* is necessary for cold nociception. Cold nociceptive defects are isoform-dependent, with *TrpA1-A* being the isoform necessary for CT response, distinct from the isoform-specific requirement of CIV-mediated high heat nociception (*TrpA1-C*). Although the isoforms required for CIV-mediated responses to chemical and mechanical stimuli are unknown, CIV neurons express only isoforms C, D, and E. *TrpA1* knockdown studies reveal a CIII cell-type-specific requirement for TRPA1 function. CIII>*TrpA1-A* rescue of CT behavior in homozygous null mutant larvae further supports the hypothesis that isoform A is functionally required in the cold nociceptor neurons for proper nocifensive behavior. While the functional role of the ARD in cold detection remains unclear, we have demonstrated that it is necessary for proper function of the channel in this sensory modality.

Consistent with our hypotheses, we have demonstrated that the *TrpA1* requirement for noxious cold-evoked behavior is independent of any roles in the establishment of dendritic morphology or downstream communication with interneurons of the CT circuit. Additionally, we have demonstrated that *TrpA1* mutants exhibit no defects in general locomotion or in responses to innocuous mechanical stimulation. This latter finding may support a functional role for *TrpA1* in CIII discrimination between innocuous mechanosensation and noxious cold detection, which evoke distinct behaviors. Although CIII>*TrpA1-A* overexpression experiments failed to reveal hyperalgesia in larvae, we hypothesize that this is the result of the already large proportion of animals that CT in response to noxious cold. Ectopic expression of *TrpA1-A* in interneurons of the adult revealed a sensitization of these otherwise non-cold-sensing neurons, suggesting that

TrpA1 is sufficient to confer cold sensitivity. Previous work has demonstrated that ectopically expressed *Drosophila TrpA1* suffices to activate neurons of interest, and it thereby serves as a thermogenetic tool (Bernstein et al., 2012). Our finding of cold sensitization may enable researchers to expand the current repertoire of available thermogenetic applications, possibly allowing for targeted activation by application of cooling stimulus in circumstances where high heat would be disadvantageous to the investigation. Live imaging with the genetically encoded Ca^{2+} sensor *GCaMP6m* and the genetically encoded Ca^{2+} integrator *CaMPARI* did not identify a role for *TrpA1* in mediating Ca^{2+} activity in response to noxious cold. However, measurement of intracellular Ca^{2+} is an indirect proxy for assessing neuronal activity, and Ca^{2+} signals are complex. While CIII knockdown of the TRP channel *Pkd2* diminishes cold-evoked Ca^{2+} transients, CIII knockdown of the TRP channel *Trpm* instead leads to an increase in Ca^{2+}, although both channels are necessary for nocifencive CT via CIIIs (Turner et al., 2016). Since multiple TRP channels operate to regulate cold-evoked CT behavior, the results obtained with *TrpA1* may be a reflection of Ca^{2+} moving through other TRPs, which may mask the effects of *TrpA1* on cold-evoked neural activity. Due to a lack of confirmatory results using these proxies, we directly measured neuronal firing via extracellular electrophysiological recordings.

We observed temperature-dependent electrical responses in control CIII neurons that collectively act to drive cold nocifensive behavior in a dose-dependent manner and are dependent upon *TrpA1*. *TrpA1* knockdown defects manifested as altered temperature-dependent firing patterns, particularly reductions in neuronal bursting upon onset of temperature change. Although numerous studies in both heterologous expression systems and lipid bilayer constructs have identified species-specific activation temperatures (Moparthi et al., 2014c, 2016), *TrpA1* has also been demonstrated to encode the rate of temperature change in *Drosophila* (J. Luo et al., 2017).

Our electrophysiological recording data are consistent with a role for rate coding in mediating larval cold nociception.

Numerous studies in heterologous systems have revealed species-specific *TrpA1* activation temperatures above ambient; these include *TrpA1* from fish, amphibians, reptiles, birds, and arthropods. As a result, the predominant view has been that ancestral *TrpA1* was high-heat-sensitive and that cold sensitivity evolved within the mammalian and fish lineages. However, these studies almost never investigated noxious low temperatures, leaving open the possibility that *TrpA1*s from diverse taxa may also be cold sensors. In this work, we hypothesized that *TrpA1*'s functional role in cold nociception is also ancestral, having arisen prior to the divergence between protostomes and deuterostomes. We conducted rescue experiments with the *TrpA1* of closely related *Drosophila mojavensis* and that of distantly related humans, observing that larval cold nocifensive behavior was rescued in both cases. Honeybee $TrpA^{AmHs}$, belonging to the *water witch* group, failed to rescue CT behavior. That the human TRPA1 channel recapitulates the function of *Drosophila* TRPA1 suggests that human and fly *TrpA1* have ancient and evolutionarily conserved functional roles in mediating noxious cold detection.

A recent and growing body of literature has implicated non-canonical receptor channels in the detection of thermal stimuli. *Gr28b*, not present in humans but expressed in both larval and adult *Drosophila*, has been shown to functionally substitute for *TrpA1* in the detection of high heat by the fly antennae (Ni et al., 2013). Microarray data from our lab revealed that *Gr28b* is enriched in CIII neurons; we therefore hypothesized that it might mediate cold detection and might genetically interact with *TrpA1* in these neurons. Mutant analyses demonstrate that *Gr28b* does in fact mediate cold nociception in larvae, and RNAi experiments confirm that this occurs via the CIII nociceptors. However, experiments with animals transheterozygously mutant for both *Gr28b*

and *TrpA1* reveal that the two channels function in parallel in these cells, rather than genetically interacting.

Reactive oxygen species are known to function in many pathways in both healthy and damaged cells (Sullivan et al., 2015; Viana, 2016), including via *TrpA1* activation. Recent work has shown that ROS is generated by noxious heat in *Drosophila*, where it mediates high heat nociception via *TrpA1* (Arenas et al., 2017). Because noxious cold has also been demonstrated to increase intracellular ROS levels (Awad et al., 2013; Sun et al., 2016), we hypothesized that high intracellular ROS would result in increased CT nocifensive behavior. Via experiments misexpressing *Duox* in CIIIs, we observed that ROS signaling facilitates cold-evoked CT behavior. Further, experiments overexpressing the ROS clearance enzymes catalase and superoxide dismutase in CIII neurons led to decreased CT response (analgesia), indicating that endogenous ROS participates in CIII-mediated cold nociception. Surprisingly, larvae expressing CIII-specific RNAi against Cat or SOD exhibited either no significant change from controls or, in select RNAi lines, impaired CT response. These results may suggest that Cat and SOD disruption result in constitutively high intracellular ROS levels, which cause damage to the cell and impair its function. On this interpretation, CIII neurons exhibit an inverted U requirement of long-term ROS levels for proper functioning.

4.1 Summary and Future Directions

Collectively, these findings demonstrate that *Drosophila TrpA1* functions in cold nociception by increasing permeability of the nociceptors to Ca^{2+}, thereby increasing neuronal firing, most notably initial bursting spikes at the onset of stimulus. We show that the functional requirement for *TrpA1* in cold nociception is likely ancestrally derived, offering insights into the

evolution of thermosensation and nociceptive mechanisms by which animals detect environmental stimuli.

Through our experiments manipulating the expression of *Duox, Cat*, and *SOD*, we have demonstrated that, whether or not *Drosophila TrpA1* is directly activated by cold stimulus below a specific threshold, larval cold nocifensive behavior is, at least in part, the result of intracellular ROS signaling. We propose the following hypotheses: 1) Noxious cold causes an increase in CIII ROS levels and, 2) ROS functions to activate TRPA1 channels. However, these hypotheses have not yet been tested directly. To evaluate whether noxious cold evokes increased ROS production, we will employ genetically encoded H_2O_2 sensors expressed CIII-specifically. We will apply a noxious cold stimulus to larvae and evaluate ROS levels via confocal microscopy. (See **Materials and Methods 2.3.5** and **2.3.9**.) We predict that H_2O_2 signal will increase under cold stimulus. In order to determine whether ROS is interacting with TRPA1 channels, we will misexpress *Duox* in the CIII neurons of *TrpA1* mutant animals. We will then perform cold plate assays and electrophysiological experiments to evaluate CT behavior and neuronal firing patterns (See **Materials and Methods 2.3.2** and **2.3.11**.) Based on our hypothesis, we predict that heightened levels of ROS will fail to evoke increased CT behavior or neuronal firing in mutant animals, as ROS will have no TRPA1 channels to activate. We will further test the hypothesis that ROS production is increased in response to noxious cold, but not innocuous mechanical, stimuli and that this distinction contributes to the CIIIs' discrimination between these two sensory modalities. In order to test this, we will perform a gentle touch mechanosensation assay on animals CIII-specifically misexpressing *Duox*. At room temperature, larvae do not CT in response to gentle touch, but we predict that increased ROS will result in larvae contracting in response to this innocuous mechanical stimulus.

We have shown that ROS acts cell-autonomously in CIIIs to facilitate CT behavior. However, whether neighboring cells generate ROS in response to noxious cold, and whether such ROS could activate CIII-expressed *TrpA1* remains unknown. To evaluate this possibility, we will misexpress *Duox* and, separately, overexpress *Cat* and *SOD* in adjacent cell types and perform cold plate assays. (See **Materials and Methods 3.3.2**.) As the CIII neurons interdigitate between muscle and epidermal tissues, these will be our target expression sites. We predict that, if ROS from adjacent cells facilitates CIII-mediated CT, we will observe increased CT in *Duox* misexpression experiments and decreased CT in *Cat/SOD* overexpression experiments.

Finally, we will test our hypothesis that dysregulation of ROS leads to cell damage, which in turn impairs neuronal activity. To accomplish this, we will express *Cat-IR* and, separately, *SOD-IR*, in CIII neurons and perform electrophysiological recordings under noxious cold stimulus. (See **Materials and Methods 2.3.11**.) We predict, based on our hypothesis, that CIII electrical activity will be significantly reduced. We will also perform optogenetic experiments as described in **Materials and Methods 2.3.6** on larvae misexpressing these RNAis. We predict that, if cell damage leads to impaired excitability/function of CIIIs, blue light stimulus will induce significantly less CT in larvae expressing *Cat-IR* or *SOD-IR* relative to control animals. Together, the results of these experiments will help to elucidate the role of ROS in noxious cold nociception and may provide a clearer understanding of the mechanisms by which polymodal neurons distinguish stimuli of different modalities.

5 SUPPLEMENTAL INFORMATION

Table 1. *Statistical tests for cold plate behavioral assays by Figure.*

Figure	Comparison	CT	NR	One-tailed Chi square; p
2-3B	*TrpA1* mutants			
	w^{1118}	24	6	
	ORR	23	7	0.098; 0.3770
	$TrpA1^{1}$	12	18	10; 0.0008
	$TrpA1^{W903*}$	6	24	21.600; <0.0001
	$TrpA1^{Xiang}$	10	20	13.303; 0.0001
2-4	Sex difference			
	w^{1118} female	19	8	
	w^{1118} male	20	10	0.090; 0.7639
	$TrpA1^{1}$ female	10	20	
	$TrpA1^{1}$ male	9	21	0.077; 0.7814
2-5A	*TrpA1-IR*			
	GAL4 control	22	8	0.082; 0.3872

	y,v	21	9	
	TrpA1-IR1 (B-31384)	10	20	8.076; 0.0022
	TrpA1-IR2 (B-66905)	11	19	6.696; 0.0048
2-5B	*TrpA1* isoform mutants			
	w^{1118}	24	6	
	$TrpA1^{\Delta A}$	10	20	13.303; 0.0001
	$TrpA1^{\Delta B}$	17	13	3.774; 0.0260
	$TrpA1^{\Delta C/D}$	17	13	3.774; 0.0260
2-5C	*TrpA1-A* rescue			
	GAL4 control (in $TrpA1^{1}$ background)	10	20	
	$TrpA1^{1}$	12	18	0.287; 0.2960
	$UAS\text{-}TrpA1\text{-}A^{Kadowaki}; TrpA1^{1}$	22	8	9.643; 0.0010
2-6B	ARD requirement			
	GAL4 control	20	10	
	w^{1118}	23	7	0.739; 0.3901
	$CIII>TrpA1\text{-}A^{\Delta ARD}$	3	27	20.376; <0.0001

2-11A	Sensitization in CIIIs			
	GAL4 control	22	8	
	UAS-TrpA1-A^{Kadowaki}	22	8	0.000; 0.5
2-11D	Proboscis extension response			
	GAL4 control	9	31	
	ORR	1	32	5.798; 0.0080
	UAS-TrpA1-A^{Xiang}	19	19	6.404; 0.0057
	UAS-TrpA1-A^{Kadowaki}	24	16	11.605; 0.0003
2-16B	Cross-species rescue			
	GAL4 control (in *TrpA1^{1}* background)	8	22	
	TrpA1^{1}	12	18	1.2; 0.1367
	UAS-TrpA1$^{D.mojavensis}$; TrpA1^{1}	20	10	9.643; 0.0010
	UAS-TrpA1^{Human}; TrpA1^{1}	25	5	19.461; <0.0001
	UAS-TrpA^{AmHs}; TrpA1^{1}	15	15	3.455; 0.0315
2-17A	*Gr28b^{c01884}* mutant			
	w^{1118}	20	10	

	Gr28b^{c01884}	8	22	9.643; 0.0010
2-17B	*Gr28b-IR*			
	GAL4 control	22	8	
	Gr28b-IR	10	20	9.643; 0.0010
2-17C	Interaction			
	w^{1118}	23	7	
	TrpA1^{1}/+	12	18	8.297; 0.0020
	Gr28b^{c01884}/+	9	21	13.125; 0.0001
	Gr28b^{c01884}/+;*TrpA1*^{1}/+	13	17	6.944; 0.0042
	TrpA1^{1}/+ vs. *Gr28b*^{c01884}/+			0.659; 0.2084
	TrpA1^{1}/+ vs. *Gr28b*^{c01884}/+;*TrpA1*^{1}/+			0.069; 0.7934
3-1	ROS misexpression			
	ORR	22	8	
	UAS-Duox	28	2	4.320; 0.0188
3-2A	Physiological ROS levels (clearance enzyme overexpression)			
	GAL4 control	22	8	

	UAS-Cat	6	24	17.143; <0.0001
	UAS-SOD (B-24750)	8	22	13.067; 0.0002
	UAS-SOD (B-33605)	6	24	17.143; <0.0001
3-2B	Physiological ROS levels (clearance enzyme *IR*)			
	GAL4 control	22	8	
	UAS-Cat-IR-1 (B-43197)	18	13	1.575; 0.1048
	UAS-Cat-IR-2 (B-34020)	9	21	11.279; 0.0004
	UAS-SOD-IR-1 (B-36804)	21	9	0.082; 0.3872
	UAS-SOD-IR-2 (B-34616)	17	13	1.832; 0.0880
	UAS-SOD-IR-3 (B-32909)	13	17	5.554; 0.0092

Table 2. *Additional statistical tests by Figure*

Figure	Passed Shapiro-Wilke Normality Test	Statistical Test Used	p value	Number of Subjects
2-3C	No	Kruskal-Wallis with Dunn's correction	>0.05	20 (w^{1118}) 23 (*ORR*) 13 ($TrpA1^{1}$) 6 ($TrpA1^{W903*}$) 11 ($TrpA1^{Xiang}$)
2-3D	No	Kruskal-Wallis with Dunn's correction	>0.05	20 (w^{1118}) 23 (*ORR*) 13 ($TrpA1^{1}$) 6 ($TrpA1^{W903*}$) 11 ($TrpA1^{Xiang}$)
2-7C	Yes	Unpaired t-test	>0.5	10 per genotype
2-7D	Yes	Unpaired t-test	>0.5	10 per genotype
2-8C	Yes	Unpaired t-test	>0.5	30 per genotype
2-8D	No	Mann-Whitney *U* test	>0.5	30 per genotype
2-9	No	Mann-Whitney *U* test	>0.5	15 (w^{1118}) 14 ($TrpA1^{1}$)
2-10	No	Mann-Whitney *U* test	>0.5	23 (w^{1118}) 18 ($TrpA1^{1}$)
2-12B	No	Mann-Whitney *U* test	>0.05	27 (*GAL4* control) 30 (*TrpA1-IR*)
2-12C	No	Mann-Whitney *U* test	>0.05	27 (*GAL4* control) 30 (*TrpA1-IR*)
2-12D	No	Mann-Whitney *U* test	>0.05	27 (*GAL4* control) 30 (*TrpA1-IR*)
2-12E	No	Mann-Whitney *U* test	>0.05	27 (*GAL4* control) 30 (*TrpA1-IR*)
2-12F	No	Mann-Whitney *U* test	>0.05	27 (*GAL4* control) 30 (*TrpA1-IR*)
2-13	No	Mann-Whitney *U* test	>0.05	48 (*GAL4* control) 45 (*TrpA1-IR*)
2-15A	No	Mann-Whitney *U* test	0.001	21 (*GAL4* control) 18 (*TrpA1-IR*)
2-15B (10°C)	No	Mann-Whitney *U* test	<0.001	21 (*GAL4* control) 18 (*TrpA1-IR*)
2-15B (15°C)	No	Mann-Whitney *U* test	<0.02	21 (*GAL4* control) 17 (*TrpA1-IR*)

Table 3. *Genotypes, Associated Figures, and Sources.*

Genotype	**Figure(s)**	**BDRC catalog number/source**
+; +; + (*OregonR*)	**2-3A**,**B**,**C**,**D**; **2-7B**; **2-11D**	B-2376
w^{*}*; +; +* (W^{1118})	**2-3A**,**B**,**C**,**D**; **2-4**; **2-5B**,**C**; **2-6B**; **2-9**; **2-10**; **2-16B**; **2-17A**,**C**	B-6326
w; +;* $TrpA1^{1}$	**2-3A**,**B**,**C**,**D**; **2-4**; **2-5C**; **2-9**; **2-10**; **2-16B**; **2-18C**	B-26504
w^{*}*; +;* $TrpA1^{W903*}$	**2-3A**,**B**,**C**,**D**	Gift of WD Tracey
w^{*}*; +;* $TrpA1^{Xiang}$	**2-3A**,**B**,**C**,**D**	Gift of Y Xiang
y^{1},v^{1}*; +; +*	**2-5A**	B-36303
+; $GAL4^{19\text{-}12}$	**2-5A**; **2-7A-D**; **2-8A-D**; **2-11A-D**; **2-12A-F**; **2-14A-D**; **2-15A-C**; **2-17B**; **3-1**; **3-2A**,**B**	Gift of YN Jan
+; UAS-Luc-IR	**2-5A**,**C**; **2-6B**; **2-7A**,**C**,**D**; **2-8A-D**; **2-12A**; **2-11A**,**B**,**D**; **2-12A-F**; **2-13**; **2-14A,C**; **2-15A-C**; **2-16B**; **2-17B**; **3-1**; **3-2A**,**B**	B-31603
UAS-TrpA1-IR; +	**2-5A**; **2-7B-D**; **2-8A**,**B,D**; **2-12A-F**; **2-13**; **2-14B,D**; **2-15A-C**	B-31384
UAS-TrpA1-IR; +	**2-5A**	B-66905
+; $TrpA1^{\Delta A}$	**2-5B**	Gift of Y Xiang

+; TrpA1$^{\Delta B}$	**2-5B**	Gift of Y Xiang
+; TrpA1$^{\Delta C/D}$	**2-5B**	B-67134
ppkGAL4,ppkGAL80; +	**2-5C**; **2-10B**; **2-16B**	Cox Lab
UAS-TrpA1-A^{Xiang}; +	**2-11D**	Gift of Y Xiang
UAS-TrpA1-A$^{\Delta ARD}$; +	**2-6A**,**A'**,**B**	Cox Lab
+; GAL4$^{19-12}$,mCD8::GFP	**2-6A**,**A'**,**B**	Cox Lab
+; GAL4^{GMR18B07}	**2-11A-D**	B-47476
UAS-TrpA1-A^{Kadowaki}; +	**2-11A**,**C**,**D**	B-61504
+; UAS-GCaMP6m	**2-12A-F**	B-42748
UAS-CaMPARI; GAL4^{677}	**2-13**	Cox Lab
+; TrpA1^{1},UAS-TRPA1$^{D.mojavensis}$	**2-16B**	B-36926
+; TrpA1^{1},UAS-TRPA1^{Human}	**2-16B**	Gift of M Gallio
+; TrpA1^{1},UAS-TRPA^{AmHs}	**2-16B**	B-36924
Gr28b^{c01884}	**2-17A**,**C**	B-10743
UAS-Gr28b-IR; +	**2-17B**	B-63545
UAS-Duox; +	**3-1**	B-77305

UAS-Cat; +	**3-2A**	B-24621
+; UAS-SOD	**3-2A**	B-24750
UAS-Cat-IR; +	**3-2B**	B-43197
UAS-Cat-IR; +	**3-2B**	B-34020
UAS-SOD-IR; +	**3-2B**	B-36804
UAS-SOD-IR; +	**3-2B**	B-34616
UAS-SOD-IR; +	**3-2B**	B-32909

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